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A PROTEIN INHIBITING THE FORMATION OF COVALENTLY LINKED COMPLEMENTARY SEQUENCES DURING DNA POLYMERASE I REACTIONS

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© WAYNE FRANCIS FLINTOFF

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance a thesis entitled "A PROTEIN INHIBITING THE FORMATION OF COVALENTLY LINKED COMPLEMENTARY SEQUENCES DURING DNA POLYMERASE I REACTIONS" submitted by WAYNE FRANCIS FLINTOFF in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

ABSTRACT

When certain DNAs are copied *in vitro* with *Escherichia coli* DNA polymerase I, the products have covalently linked complementary sequences. These sequences are thought to be caused by the polymerase either switching template strands at the growing fork, or turning back at the end of a strand. A protein that prevents the accumulation of such sequences during DNA synthesis has been purified from *Escherichia coli*. We have called this protein S factor. The purification procedure involves autolysis, gel exclusion chromatography, denaturation-renaturation, and ion exchange chromatography.

The S factor activity is heat labile, contains no detectable nuclease activity, and migrates as a single band of apparent molecular weight 11,500 in SDS-acrylamide gel electrophoresis.

The S factor was effective in blocking the accumulation of covalently linked complementary sequences during the synthesis of the defined DNA $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ and to a limited extent during the copying of *Escherichia coli* DNA. Once covalently linked complementary sequences were formed, however, S factor was unable to remove them. Two mechanisms are discussed whereby S factor could prevent the formation of such sequences during DNA synthesis: the first, by an endonuclease nick at the point where strand-switching has occurred; the second, more likely, by binding to the displaced DNA strand and preventing strand-switching.

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LIST OF ABBREVIATIONS

AMV	avian myeloblastosis virus
ATP	adenosine 5'-triphosphate
dATP	deoxyadenosine 5'-triphosphate
dC	deoxycytosine
CCC	covalently closed circular
CLC	covalently linked complementary
cm	centimeter
cpm	counts per minute
CMP	cytidine 5'-monophosphate
CTP	cytidine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
DEAE-	diethylaminoethyl-
DNA	deoxyribonucleic acid
DNase I	bovine pancreatic deoxyribonuclease I
DS	double stranded
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetracetate
g	grams
M	molecular weight
mg	milligram
ml	milliliter
mm	millimeter
ng	nanograms
nm	nanometer
nmoles	nanomoles

3'-dNMPs	deoxynucleoside 3'-monophosphates
r(d) NTPs	ribo (deoxy) nucleoside 5'-triphosphates
OD	optical density
Pol A	amber mutation affecting *E. coli* DNA polymerase I
RF	replicative form
RNA	ribonucleic acid
rpm	revolutions per minute
S	strand separability (factor)
$S_{20,w}$	sedimentation coefficient corrected to 20° for solvent viscosity and density
$S^{\circ}_{20,w}$	intrinsic sedimentation coefficient
SDS	sodium dodecyl sulfate
SS	single-stranded
T	thymine
T_m	melting temperature
tRNA	unfractionated yeast transfer ribonucleic acid
Tris	tris (hydroxymethyl) aminomethane
TTP	thymidine 5'-triphosphate
UV	ultraviolet
V_e	elution volume
V_o	void volume
µg	micrograms
µl	microliters
ρ	density

All temperatures are in degrees Centigrade.

CHAPTER I

INTRODUCTION

A great deal of information on the structure of DNA has accumulated since Watson and Crick proposed the double-helical concept (1). Studies with bacterial and viral DNAs have been particularly useful in providing details of the structures of replicating DNAs.

In replication, the two complementary chains of the parental DNA duplex separate permanently, and are transferred, one to each of the progeny chromosomes. Radioautographs of replicating E. coli DNA (2) indicate that the replication occurs within a circular molecule and seems to be accomplished at a fork that moves along the structure, leaving two separated daughter helices behind. Additional information on the structures of replicating DNAs has come from electron micrographs of smaller DNAs:

(1) Replication frequently involves a circular molecule. For example, intermediates in the DNA replication of λ phage (3), P2 phage (4), 186 phage (5), SV40 virus (6), and mitochondria (7) are circular. However, for T7 phage DNA (8) and adenovirus 5 DNA (9) linear replicating intermediates are found.

(2) The Y-shaped growing fork often contains single-strand connections (Figure 1a). These single-stranded regions occur only in one of the daughter strands at a growing fork (3). For λ DNA,

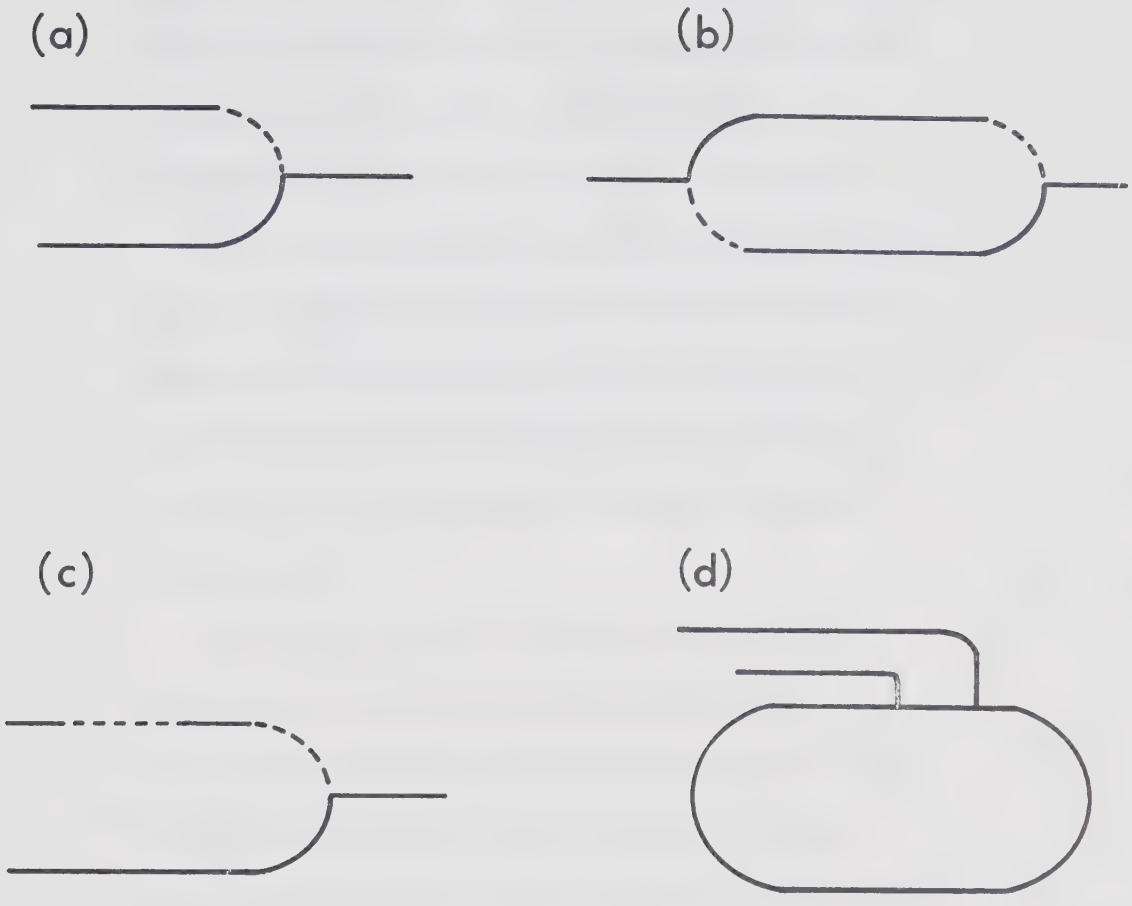


FIGURE 1. Diagramatic Representations of <u>In Vivo</u> DNA Replicating Structures.

———, double-stranded; - - - - -, single-stranded.

such regions are less than 1% the length of mature DNA (3). In cases where two growing forks occur in the same molecule [λ phage DNA (3) and T7 phage DNA (8)], these single-strand connections occur only in "trans" (Figure 1b).

(3) Single-stranded regions not associated with the growing fork are also observed in an otherwise double-stranded branch (Figure 1c). Such structures have been seen in the DNAs of λ phage (3), T7 phage (8), T4 phage (10), and 186 phage (5).

(4) Initiation of replication occurs at an unique site on the chromosome and can be physically located by denaturation mapping. For λ DNA this physical origin (11) is in good agreement with the origin established by other techniques (12). In some cases [for example, T4 phage DNA (10) and 186 phage DNA (5)], it appears that a second round of synthesis can begin before the first has terminated. The result is a multi-branched structure in which the branches are of varying lengths (Figure 1d).

(5) Replication in one direction [for example, the DNAs of phage 186 (5), phage P2 (4), and mitochondria (13)] or both directions [as for λ phage (3), T7 phage (8), and T4 phage DNAs (10)]

from this origin have been observed. Whether the unidirectional replication represents asymmetric bidirectional replication, in which the replication in one direction is stopped shortly after initiation, is not known.

The single-stranded nature of the replicating intermediates suggests that copying of at least one branch of the replication fork may be by a discontinuous mechanism (Figure 2).

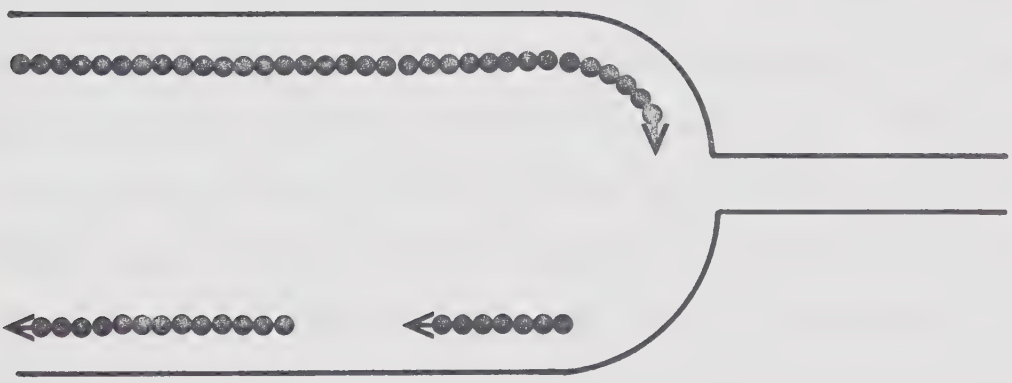


FIGURE 2. A Model for Discontinuous Synthesis.

———, parental DNA; ●●●●, new DNA, with the arrowhead designating the 3', growing end.

Consistent with a discontinuous mechanism is the finding that a certain portion of nasent DNA can be isolated as relatively low molecular weight chains, 'Okazaki' fragments, after alkali denaturation (14,15,16,17,18). For T4 DNA, these fragments were shown to be growing at the 3' end (18), suggesting that synthesis occurs in the 5'→3' direction. The initiation of synthesis on the daughter strand which has its direction of growth opposite to the direction of fork movement (lower strand Figure 2) may be RNA primed (16,19,20). For E. coli DNA, two molecular weight classes of Okazaki fragments, which are mutually but not self-complementary, have been identified (67). This finding suggests that DNA synthesis may be discontinuous on both strands.

The system responsible for replication involves the co-operative activities of numerous gene products. For example, some 19 T4 genes are required for DNA production (21). In E. coli, there are at least 7 such genes (dna A to G) (22). A limited number of these gene products has been identified. One required function is a DNA polymerizing activity, since a mutation in the structural gene for T4 DNA polymerase (gene 43) (23) or T7 DNA polymerase (gene 5) (24) is lethal. Identification of the polymerizing activity in E. coli has been complicated by the existence of three such enzymes. Genetic evidence indicates that E. coli DNA polymerase III (dna E locus) is required for chromosomal replication (25). The other polymerases may be involved in other forms of replicative synthesis. For example, E. coli DNA polymerase I has been shown to be required for the replication of colicinogenic factor E1 (26,27), and is implicated in elongation, or "finishing" of Okazaki fragments (28,29). When all three activities are present (as in a wild type cell), there is no indication which one is responsible for the DNA synthesis. Recently, two additional

gene products required for phage DNA replication have been purified. These proteins -T4 gene 32 protein (30) and fd phage gene 5 protein (31) - bind co-operatively to single-stranded DNA *in vitro*. In addition, the T4 gene 32 protein specifically stimulates DNA synthesis catalyzed by T4 DNA polymerase *in vitro* (32). Whether these *in vitro* activities are measures of their *in vivo* activities (Table I) is still unproven. Recently, the *E. coli* dna C (37) and dna G (38) gene products have been purified but their enzymatic functions in DNA synthesis are unknown. The *E. coli* dna F locus codes for ribonucleotide reductase (39).

Several proteins from various sources possess activities *in vitro* which suggest a role for these proteins in replication. Table II lists some of these proteins and summarizes their *in vitro* activities. In addition, evidence from both *in vivo* (47,48) and *in vitro* (49,50) experiments suggests a primer role for RNA in replication. In some cases, this RNA appears to be synthesized by a known RNA polymerase (47), and in others (48) not. Although several protein components may be implicated in DNA replication their true involvement awaits genetic evidence.

Two systems which hold great potential not only for the identification of replication components but also for the reconstruction of the replication process are:

(1) the permeable cell system (51,52) in which *E. coli* cells are made permeable to a wide range of compounds, including some macromolecules, by treatment with organic solvents, and

(2) the Cellophane membrane disc system (53) in which the macromolecular cellular components,

TABLE I

PROPERTIES OF T4 GENE 32 PROTEIN AND fd PHAGE GENE 5 PROTEIN

Protein	*In vivo* Requirement	*In vitro* Activity
T4 gene 32 protein	Required in stoichiometric amounts throughout the infectious cycle for both DNA recombination and replication (33,34).	Binds co-operatively to single-stranded DNA, lowers the melting temperature of certain DNAs (30), and specifically stimulates DNA synthesis catalyzed by T4 DNA polymerase (32).
fd gene 5 protein	Required for synthesis of single-stranded circles from progeny doubled-stranded (RF) templates (35,36).	Binds co-operatively to single-stranded DNA, and lowers the melting temperature of several DNAs (31).

TABLE II

PROTEINS THAT MAY BE INVOLVED IN DNA REPLICATION

Protein	Source	*In vitro* Activity	Speculated *In vivo* Activity
Polynucleotide Ligase	*E. coli* (40)	Seals single-stranded breaks in double-helical DNA if the two adjacent single-stranded ends exhibit a free 3'-hydroxyl and a 5'-phosphate group respectively.	May seal the short DNA fragments that would be generated by a discontinuous mode of replication.
	T4 (41)		
"ω" Protein	*E. coli* (42)	Relaxes negative superhelical turns in a closed-circular DNA resulting in a non-twisted DNA that is still covalently closed.	Could act to relieve the topological strain that results from unwinding circular DNA at the replication fork.
	Mouse Embryo (43)	Relaxes both positive and negative superhelical turns in a closed-circular DNA yielding a covalently closed untwisted molecule.	

TABLE II (Continued)

PROTEINS THAT MAY BE INVOLVED IN DNA REPLICATION

Protein	Source	*In vitro* Activity	Speculated *In vivo* Activity
Single-stranded DNA Binding Proteins	*E. coli* (44)	Binds co-operatively to single-stranded DNA, lowers the melting temperature of DNAs, and specifically stimulates the DNA synthesis catalyzed by *E. coli* DNA polymerase II.	May help to unwind the parental strands at the replication point.
	T7 (45)	Binds co-operatively to single-stranded DNA, and specifically stimulates the DNA synthesis catalyzed by T7 DNA polymerase.	
	Calf Thymus (46)	Binds co-operatively to single-stranded DNA.	

after cell lysis, are maintained at a concentration approaching their *in vivo* level.

Any system which allows the identification of components affecting *in vitro* DNA synthesis may be useful for understanding replication. One such system is the synthesis catalyzed by *E. coli* DNA polymerase I *in vitro*. Polymerase I provides a reasonable model for enzymes which replicate DNA for the following reasons:

(1) All template-dependent polymerases isolated to date, whether of viral (24,54), bacterial (55,56,57,58) or mammalian (59) origin perform a "repair-type" synthesis *in vitro*. That is, they add deoxynucleotide residues to the 3'-hydroxyl terminus of a primer molecule under the direction of a single-stranded template to which the primer is annealed.

(2) The replication of colicinogenic factor E1 appears to require *E. coli* DNA polymerase I (26,27).

(3) The amber mutations of *E. coli* DNA polymerase I (Pol A) retain measurable levels of polymerase I activity (60). A deletion mutation affecting polymerase I has never been isolated, suggesting that this mutation may be lethal. If such a mutation were isolated and chromosomal replication unaltered, this would establish that polymerase I is not essential for replication. However, until such a

mutant is isolated, a role for polymerase I in chromosomal replication cannot be excluded.

A property of the DNA product synthesized by E. coli DNA polymerase in vitro is that the complementary daughter strands are covalently linked. Such DNA will be referred to as CLC (for covalently linked complementary) DNA. This was shown for the copying of natural DNAs by Schildkraut, Richardson, and Kornberg (61). A subsequent report indicated that CLC sequences also appeared during the copying of the chemically defined polymer $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ (62). This mode of synthesis presumably involves either strand switching at the growing point (Figure 3a), or turning back at the end of a strand (self-copying) (Figure 3b).

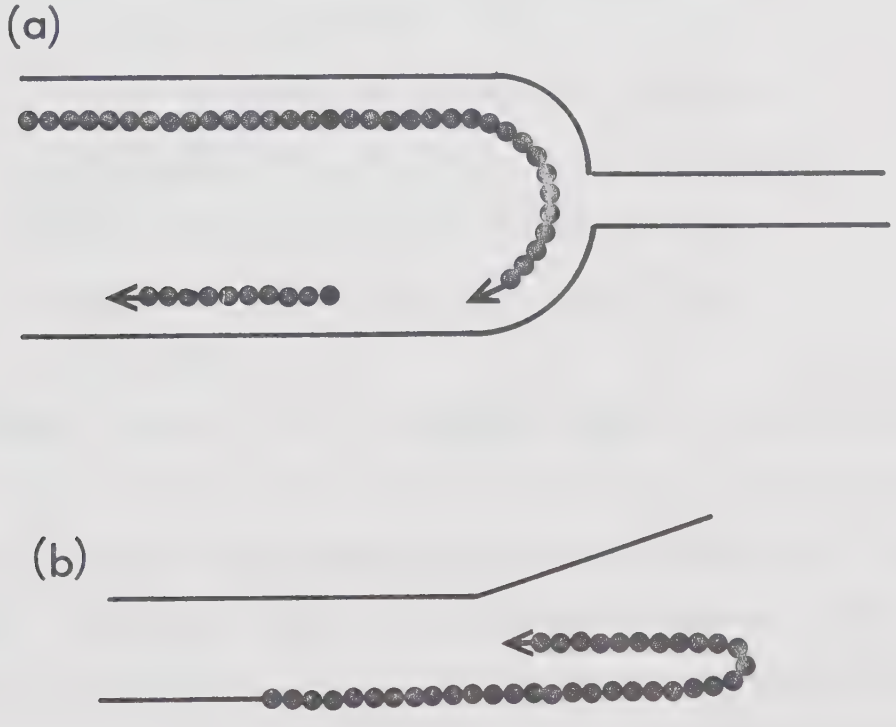


FIGURE 3. Mechanisms for CLC DNA Production.

———, parental DNA; ●●●●, new DNA, with the arrowhead designating the 3', growing end.

There are several reports that CLC structures may be intermediates during DNA synthesis *in vivo*:

(1) Electron micrographs of replicating colicin E1 DNA show superhelical circles with double-stranded tails (63). These structures can be interpreted to indicate covalent continuity between the complementary daughter strands at the growing fork. If the molecules were nicked at the growing fork, the circles would have been relaxed.

(2) Lambda DNA at some stage of its development contains covalent linkers between the daughter strands (64).

(3) Newly made T7 DNA has been isolated as renaturing structures (65). This observation, however, has not held up in our laboratory (Langman, L. and Paetkau, V.H.: unpublished observations).

Other evidence indicates that in *Bacillus subtilis* the CLC sequences are not formed from the newly made DNA (66). Thus, CLC structures may exist as replication intermediates in some systems, under some conditions. They would provide primers for the daughter strand which has its direction of growth opposite to the direction of fork movement (Figure 2). However, it appears that initiation can occur on either strand (67), thus there would be no requirement for CLC structures.

An earlier report described the ability of crude DNA polymerase fractions to block the accumulation of CLC sequences during the polymerase I mediated copying of a defined DNA *in vitro* (62). A factor having this activity could either remove CLC linkers, if in fact these exist as normal replication intermediates, or else prevent their formation during polymerase mediated DNA copying *in vitro*. The purification and partial characterization of this activity, which we have called S factor for separability of strands (that is, absence of CLC sequences) is the subject of this thesis.

CHAPTER II

GENERAL MATERIALS AND METHODS

I. Materials

A. Chemicals

Unlabelled ribo- and deoxynucleotides were purchased from PL Biochemicals; radioactive (^{14}C and ^{3}H) ribo- and deoxynucleoside triphosphates were from Schwarz-Mann; [α-^{32}P] dATP was from International Chemical and Nuclear Corporation. Cesium chloride was from Pierce Chemical Corporation or Schwarz-Mann. Ethidium bromide was obtained from Sigma Chemical Company.

Agarose 15M (200-400 mesh) and 5M (200-400 mesh) were purchased from Bio-Rad Laboratories. Phosphocellulose (P11) and DEAE-cellulose (DE-23) were from Whatman Industries. Sephadex G-25 (20-80 μ), G-50 (20-80 μ), G-75 (40-120 μ), DEAE-Sephadex (A25), and Blue Dextran 2000 were from Pharmacia Fine Chemicals.

Gel electrophoresis supplies were obtained from Eastman Organic Chemicals.

All other chemicals were reagent grade and were used without further purification or treatment.

B. Biological Materials

Marker proteins (ovalbumin and chymotrypsinogen A) were from Pharmacia Fine Chemicals. Sperm whale myoglobin and cytochrome c were gifts from Dr. Jutta Seehafer; R17 phage, a gift from Mrs. L. Frost.

Pancreatic deoxyribonuclease I (DNase I), micrococcal nuclease, and spleen phosphodiesterase were purchased from Worthington Biochemical Corporation. E. coli B cells, grown to 3/4 log phase in minimal medium, were purchased frozen from Grain Processing Company, Muscatine, Iowa.

C. Nucleic Acids

The defined DNAs with repeating sequences have already been described (68). The PM2 DNA and the polymer $d(T\text{-}C\text{-}C)_n \cdot d(G\text{-}G\text{-}A)_n$ were gifts from Dr. A.R. Morgan. Unfractionated yeast transfer RNA (tRNA) was a gift from Dr. C.J. Smith.

II. Methods

A. Reagents

Buffers and solutions were millipore filtered with an HAWP 04700 membrane before use.

Resins were prepared according to the procedures described by the manufacturers.

B. Radioactivity

Radioactivity was measured with a Beckman LS-250 liquid scintillation spectrometer using a toluene-based scintillator (14.4 g 'Omnifluor', New England Nuclear, in 3.8 liters of toluene). Restricted channels were used for double-labelled experiments and the counts were corrected for a 25% overlap of ^{14}C into the ^{3}H channel. Specific activities of labelled deoxynucleoside triphosphates were determined in Aquasol (New England Nuclear).

C. Fluorescence

Fluorescence of ethidium bound to DNA was measured with a Turner spectrofluorometer Model 430 at an excitation wavelength of 525 nm and an emission wavelength of 600 nm. The temperature was maintained at 25° by a circulating water bath. Because of fluctuations in xenon lamp intensity, the instrument was periodically calibrated with a known concentration of DNA in ethidium (69).

D. Protein Determinations

Protein concentrations were determined either by the modified Biuret assay (70), or by the absorbance at 280 nm.

E. Purification of E. coli DNA Polymerase I

E. coli DNA polymerase I was purified by the method of Jovin, Englund, and Bertsch (71) with the following modification. Fraction 7 (Sephadex G-100 fraction) was subjected to an additional phosphocellulose chromatography step (Fraction 8), using the conditions required to obtain Fraction 6 enzyme. Essentially all of the protein had the mobility of DNA polymerase in SDS-gel electrophoresis, 85% as the 110,000 molecular weight form and the rest as the 76,000 molecular weight form (72,73). The specific activity was 10,000 $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$ units per mg of protein (1 unit being defined as the incorporation of 10 nmoles of total acid-insoluble nucleotide per 30 minutes at 37°).

F. Fraction DIII S Factor

DIII was obtained as a side product of a modified (74) Chamberlin and Berg RNA polymerase preparation (75). Material extracted

from the protamine pellet with 10 mM Tris-Cl, pH 8.0, 0.1 M $MgCl_2$, 0.1 mM EDTA, and 10 mM β-mercaptoethanol was fractionated with ammonium sulfate and the fraction precipitating between 45% and 65% saturation redissolved in 0.02 M potassium phosphate buffer, pH 7.4, to yield DIII (protein concentration 1.8 mg/ml).

G. Fraction 4 DNA Polymerase I and S Factor

Fraction 4 was the same as Fraction IV obtained from the DNA polymerase I purification procedure (76) except that the ammonium sulfate precipitate was dissolved in a smaller volume of 0.02 M potassium phosphate, pH 7.2, yielding a protein concentration of about 40 mg/ml.

H. Purification of E. coli DNA

E. coli DNA was isolated by the method of Marmur (77). It was further purified by heating to 47° for 15 minutes in the presence of 0.1% SDS and chromatography on a 5M Agarose (200-400 mesh) column (6.15 cm^2 x 45 cm) equilibrated with 10 mM Tris-Cl, pH 8.0, and 0.1 mM EDTA. The excluded high molecular weight DNA was used as template.

I. In vitro DNA Synthesis

The basic procedures for in vitro DNA synthesis were as described by Morgan, A.R., Coulter, M.B., Flintoff, W.F., and Paetkau, V.H. (manuscript submitted). The standard mixture for enzymatic synthesis of defined DNAs contained: 30 mM potassium phosphate, pH 7.4; 12 mM $MgCl_2$; 2 mM dithiothreitol; 5.2 mM total of the four deoxynucleoside triphosphates, in the ratio of their frequency in the DNA being

synthesized; DNA template at 0.2 OD_{260}; 6 µg/ml Fraction 8 DNA polymerase I; and S factor as indicated. Tritium and ^{14}C labelled dNTPs were present as indicated. Incubations were at 37°. When DIII, or Fraction 4, or S factor Fractions 5, 6 or 7 were present, 0.5 OD_{260} tRNA was also added to inhibit endonuclease I (78). Pancreatic DNase I was added to stimulate synthesis. Figure 4 shows the effect of various DNase I levels on the molecular weight and synthesis of $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$. Pancreatic DNase I at 25 ng/ml resulted in a reasonable synthetic rate without an appreciable reduction in molecular weight. Under these conditions, 25- to 40-fold copying of the defined templates occurred in 5-6 hours.

Defined DNAs were characterized, when required, by their neutral buoyant densities in CsCl, and their melting temperatures (T_ms).

The conditions for the <u>in vitro</u> synthesis of natural DNAs were the same as for defined DNAs except that the initial template was 0.5 or 1 OD_{260} and the potassium phosphate was 67 mM.

The extent of synthesis was measured either by the incorporation of radioactive deoxynucleoside triphosphates into acid insoluble material by a modification (74) of the filter paper disc method (80), or by the enhanced fluorescence of ethidium bromide when bound to bihelical DNA, as described in the fluorescence assay for covalently linked complementary DNA.

J. Isolation of DNAs

Polymers were deproteinized and separated from small molecules by a modification of an earlier method (81). The reaction was stopped by the addition of EDTA (final concentration twice that of $MgCl_2$)

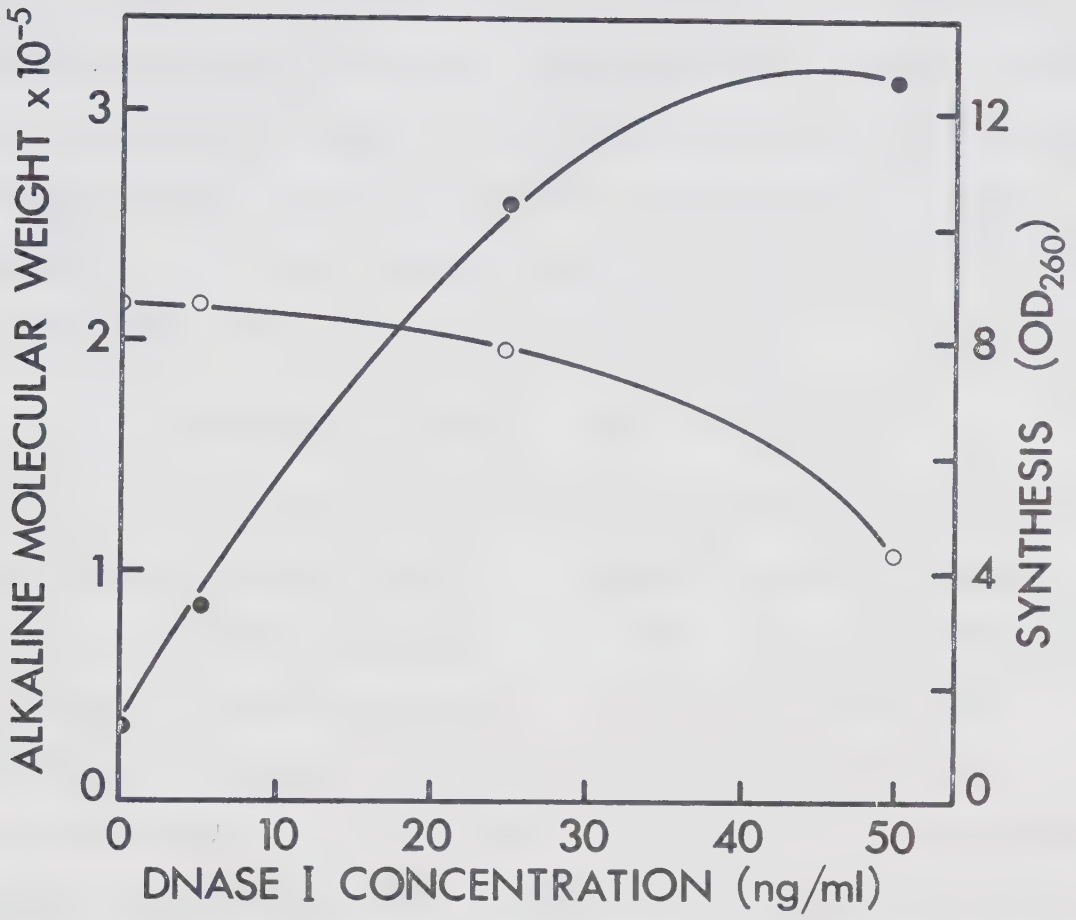


FIGURE 4. The Effect of Pancreatic DNase I on the Synthesis and Molecular Weight of $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$.

Polymers were synthesized under standard conditions with 36 µg/ml DIII, 0.5 OD_{260} tRNA, and various concentrations of pancreatic DNase I. Incubations were for 6 hours. Portions were removed and synthesis determined by the fluorescence method. The polymers were isolated by Agarose chromatography and concentrated by vacuum dialysis. Their molecular weights were determined by the Studier method (79).

○———○, molecular weight in alkali; ●———●, synthesis as monitored by ethidium bromide fluorescence.

and the solution made 0.1% in either SDS or sodium dodecyl sarcosinate (Sarkosyl). Samples were chromatographed at room temperature on 15M Agarose (200-400 mesh) columns (0.63 cm^2 x 30 cm) equilibrated with 5 mM Tris-Cl, pH 8.0, 20 mM NaCl, and 0.1 mM EDTA. A typical elution pattern is shown in Figure 5. The DNA appeared in the excluded peak separated from the proteins, tRNA, SDS, deoxynucleotides, and salts. The DNA was concentrated against 5 mM Tris-Cl, pH 8.0, and 0.1 mM EDTA by vacuum dialysis using a Sartorius collodion bag.

K. Preparation of $^{3}H\text{-}d(\overset{*}{T}\text{-}G)_n$ and $^{14}C\text{-}d(\overset{*}{C}\text{-}A)_n$

Labelled $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ was prepared under standard conditions with 36 μg/ml DIII, ^{3}H-TTP (specific activity 2700 cpm/nmole), and ^{14}C-dCTP (specific activity 1600 cpm/nmole). After 5 hours of synthesis the sample was added to a solution of alkaline CsCl (0.05 M NaOH, 1 mM EDTA) such that the final density (determined refractometrically) was 1.760 g/cm^3. The solution was overlayed with silicone oil (Corning DC 556), and centrifuged to equilibrium in a Ti 50 rotor using a Beckman L2-65B ultracentrifuge. Conditions of centrifugation were 38,000 rpm, at 20°, for 65 hours. A needle was lowered into the gradient and the material pumped out with a peristaltic pump. The fractions containing the separated strands were located by their radioactivity. The regions corresponding to the separated strands were pooled, neutralized, and concentrated by vacuum dialysis against 5 mM Tris-Cl, pH 8.0, and 0.1 mM EDTA.

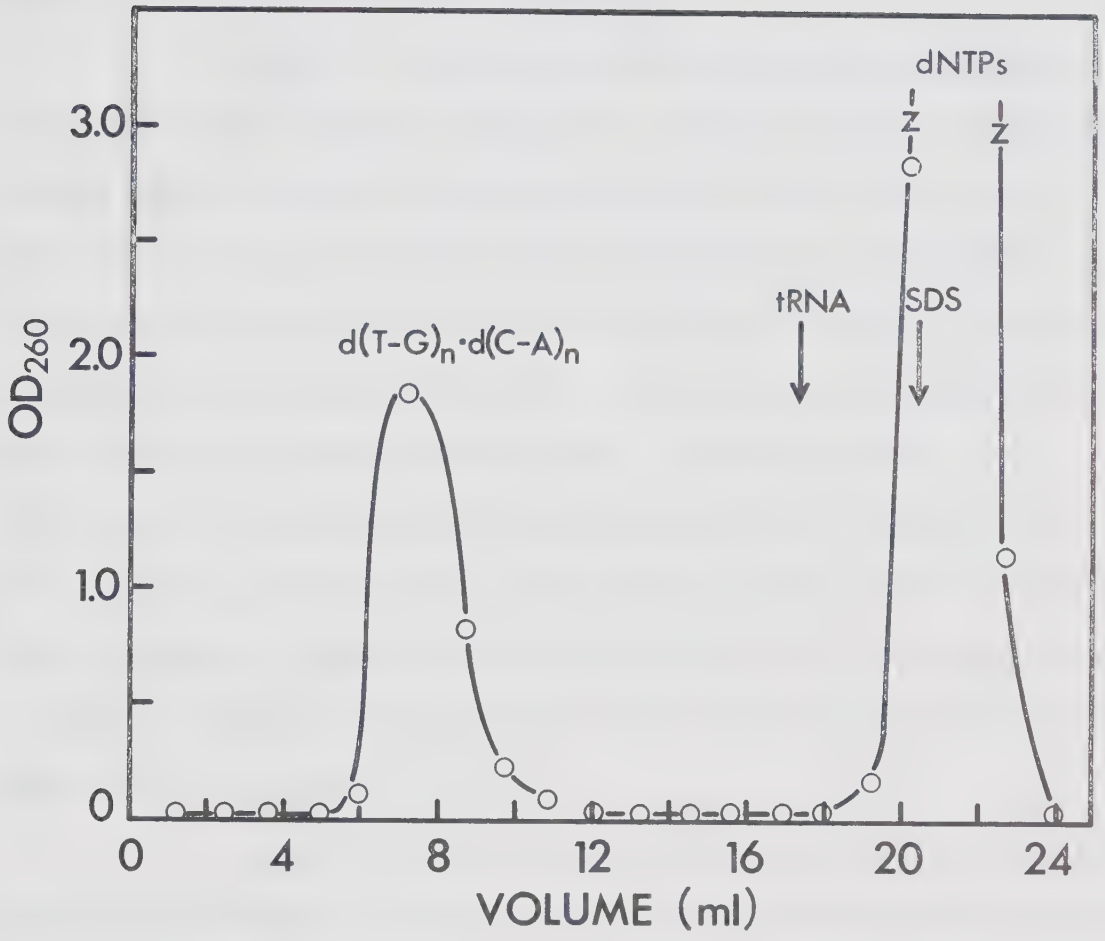


FIGURE 5. Agarose 15M Gel Filtration of $d(T-G)_n \cdot d(C-A)_n$.
A DNA polymerase reaction synthesizing $d(T-G)_n \cdot d(C-A)_n$ was treated as indicated in the text. The sample was applied to a 15M Agarose (200-400 mesh) column (0.63 cm^2 x 30 cm) equilibrated with 5 mM Tris-Cl, pH 8.0, 20 mM NaCl, and 0.1 mM EDTA. Fractions were 1.2 mls. A separate run determined the elution of 3.5 OD_{260} units of tRNA.
○———○ , OD_{260}.

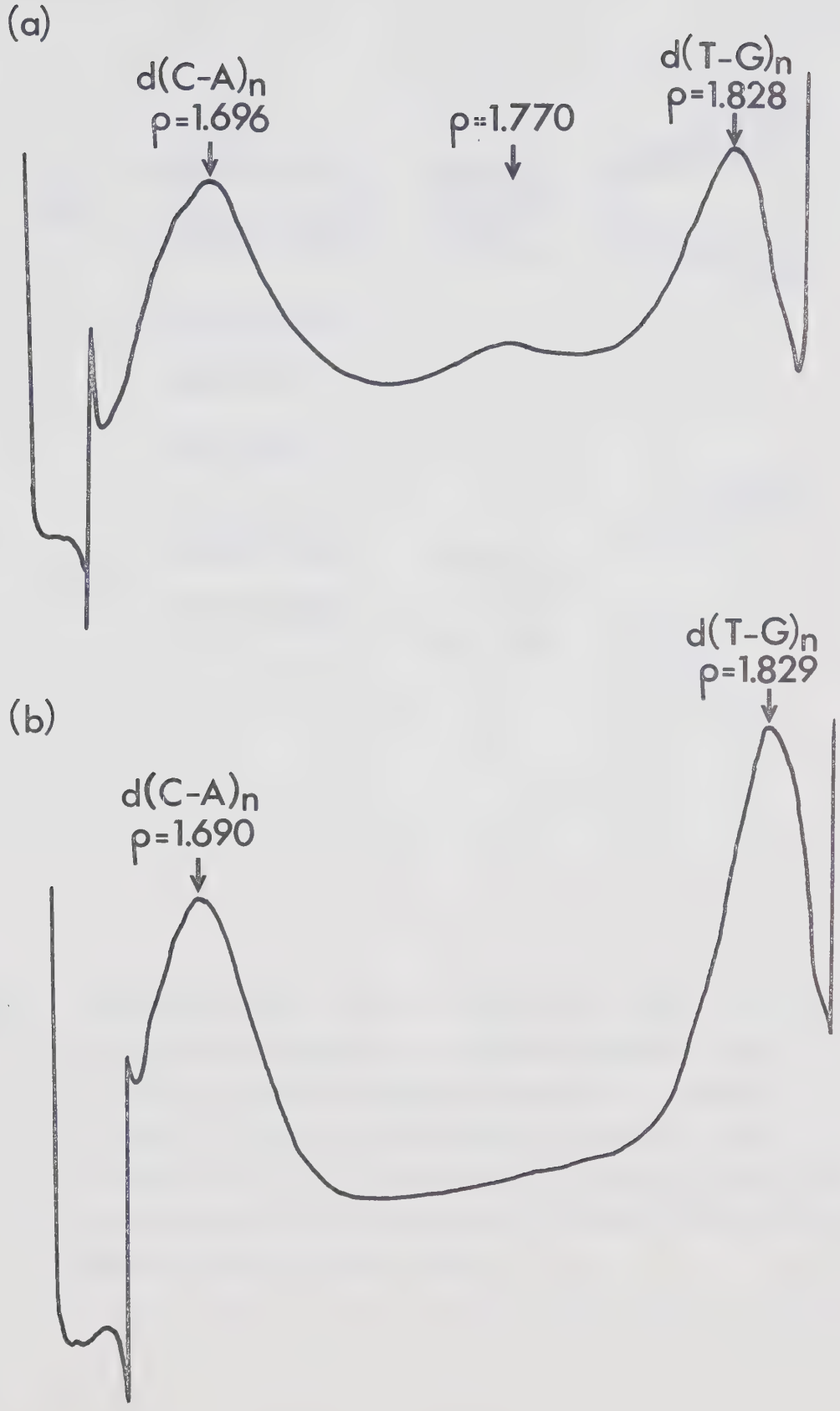
L. Assays for S Factor Activity

Since S factor prevented the production of CLC DNA, measurements of S factor activity involved assays for CLC DNA. CLC DNA was measured by the following techniques.

Assay 1. Alkaline CsCl equilibrium density banding is a semi-quantitative technique applicable to DNAs in which the complementary strands can be physically separated [such as $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ and $d(T\text{-}C\text{-}C)_n \cdot d(G\text{-}G\text{-}A)_n$]. Polymers were synthesized under standard conditions in the absence or presence of various S factor fractions, isolated by Agarose chromatography, and banded in alkaline CsCl according to the method outlined by Wells and Blair (82). Figure 6a shows a $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ polymer made in the absence of DIII; Figure 6b, a $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ polymer made in the presence of DIII. The intermediate density material, containing both $d(T\text{-}G)_n$ and $d(C\text{-}A)_n$ and characteristic of covalently linked complementary sequences, was absent in the polymer made with DIII present.

Assay 2. The fluorescence assay is a quantitative method based on the enhanced fluorescence of ethidium bromide when bound to bi-helical DNA (69,84,85). The rationale of this method is shown in Figure 7. After heat denaturation at low ionic strength, ionic repulsion keeps the separated DNA strands apart (Figure 7b). However, if a covalent linker is present (Figure 7a), it acts as a nucleation site for strand reannealing. In this assay, either isolated polymers or portions removed directly from a DNA synthesizing system were used, since none of the components of the DNA polymerase reaction interfered with the fluorescence. Duplicate portions were removed such that the DNA was between 0.01 and 0.04 OD_{260} in the assay. The samples were

FIGURE 6. Analytical CsCl Gradient Ultracentrifugation of $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$.
Polymers were synthesized either with (b) or without (a) 36 µg/ml DIII, isolated by Agarose chromatography, and centrifuged to equilibrium in alkaline CsCl gradients. Conditions of centrifugation were 48,000 rpm, at 25°, for 22 hours. Densities were determined by the isoconcentration method (83).


(a)
d(C-A)n
ρ=1.696
ρ=1.770
d(T-G)n
ρ=1.828
(b)
d(T-G)n
ρ=1.829
d(C-A)n
ρ=1.690

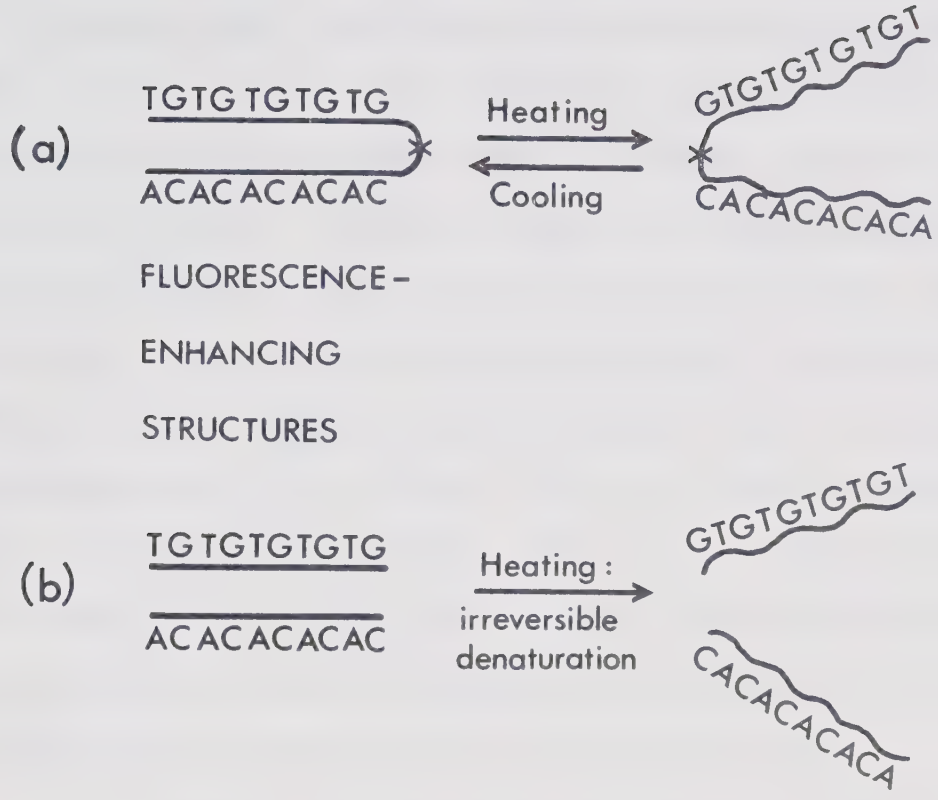


FIGURE 7. Ethidium Bromide Assay for Covalently Linked (CLC) DNA. At low ionic strength, CLC sequences renature after heating and quick cooling (a) because of a covalent linkage, X, between complementary sequences. This linkage acts as a nucleation site for strand reannealing. Non-CLC sequences (b) irreversibly denature upon heating because they lack such a site.

diluted into 3 mls of either TE buffer (2 mM Tris-Cl, pH 8.4, 0.2 mM EDTA) for defined DNAs, or KE buffer (20 mM K_3PO_4, pH $\sim$12, 0.2 mM EDTA) for natural DNAs. The KE buffer destroys the relatively non-specific structures observed with denatured, non-CLC natural DNAs (69). One portion was heated in boiling water for 5 minutes and quickly cooled in an ice slurry. Ethidium bromide (final concentration 0.5 µg/ml) was added to both portions and the fluorescence measured. When related to the fluorescence of standard DNA samples, the fluorescence of the unheated portion measured the total bihelical DNA; the fluorescence of the heated portion, the CLC DNA. The ratio of these two measurements gave the percentage of the total DNA which was CLC. Table III gives the CLC content of the $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ polymers described in Figure 6. Polymer made in the absence of Fraction DIII had a CLC content of 25%; polymer made in the presence of DIII, a CLC content of 4%.

Assay 3. Nearest neighbor analysis is a third technique for determining CLC DNA. Since the polymers were copied with great fidelity overall, the occurrence of CLC linkers between complementary, defined sequences would necessarily lead to "wrong" nearest neighbors. Polymers were copied in the presence of [α-^{32}P] dATP with DNA polymerase I and Fraction DIII was added as indicated. Isolated polymers were digested overnight at 37° with 0.2 µg/ml micrococcal nuclease in 10 mM glycine buffer, pH 9, containing 2 mM $CaCl_2$, then for several hours with 50 µg/ml spleen phosphodiesterase in 100 mM ammonium acetate, pH 5.9. Samples were evaporated to dryness, taken up in 1/10 volume of carrier 3'-dNMP mixture, applied to Whatman 3MM paper, and electrophoresed in 0.1 M sodium citrate, pH 3.5, for 3 hours at 30 volts/cm.

TABLE III

DETERMINATION OF CLC SEQUENCES IN $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ BY VARIOUS TECHNIQUES

Conditions of Synthesis	Fluorescence CLC Assay	Nearest Neighbor dNpdA N = A, = G, = T	CsCl Gradient
Polymerase alone	25%	3.1%, 7.8%, 3.7%	Figure 6a
Polymerase + 36 µg/ml DIII	4%	< 0.6%, < 1.0%, < 1.9%	Figure 6b

The paper was dried, cut into 1 cm strips, and counted. Table III shows a nearest neighbor analysis of the $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ polymers described in Figure 6. The polymer made in the absence of DIII contained a significant level of "wrong" nearest neighbors to dA compared to polymer made with DIII present.

All three methods gave consistent and complementary information on the presence of CLC sequences. The low level (4%) of CLC sequences observed with DIII present when the fluorescence, but not the ultracentrifuge assay was used, suggests a real difference between these methods. The difference may arise from low molecular weight CLC sequences which register fluorescence but do not form discrete bands in the ultracentrifuge.

For assaying S factor activity routinely, the method of choice was the fluorescence assay. High levels of non-specific nucleases will reduce the apparent CLC DNA by generally degrading the DNA. Therefore, the presence of CLC DNA when high levels of nuclease were present was determined by the ultracentrifuge method.

The routine template for determining S factor activity was $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$. The polymer was synthesized under standard conditions in the absence or presence of various S factor fractions. At the end of 5 hours, portions were removed from the incubation mixture and CLC DNA content determined by the fluorescence assay. A 50% decrease in the CLC content of $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ was defined as one unit of S factor activity.

M. Analytical Ultracentrifugal Analyses

Equilibrium banding experiments were performed in a Beckman Model E ultracentrifuge with UV optics, using a 4°, 12 mm Kel-F centerpiece with a -1° wedge window. The solution contained DNA at 0.15 OD_{260}, 1 mM EDTA, 20 mM NaOH, and CsCl to the required density. Centrifugations were at 48,000 rpm for at least 20 hours at 20°. Densitometric tracings of photographs taken at equilibrium were made with a Joyce-Loebel microdensitometer. The density of the DNA after equilibrium had been reached was calculated by the isoconcentration method (83) using published β values (86).

Molecular weights were determined from sedimentation velocity runs in the Beckman Model E ultracentrifuge using the method of Studier (79). Band sedimentation was done with a Vinograd type 1 cell, a 4°, 12 mm Kel-F centerpiece with quartz windows. The DNA (20 µl of 2 OD_{260}) was layered at low speed onto the bulk solvent and centrifuged at 56,000 rpm and 25°. Photographs were taken at 8 minute intervals. For sedimentation at neutral pH, the solvent contained 1 M NaCl, 0.035 M Na_2HPO_4, 0.015 M NaH_2PO_4, and 0.1 mM EDTA, pH = 6.7. For sedimentation at alkaline pH, the solvent was 0.9 M in NaCl, 0.1 M in NaOH, and 0.1 mM in EDTA. In the latter case, the sample was made 0.2 M in NaOH. The sedimentation coefficient was calculated and corrected for solvent viscosity, temperature, and density using the correction factors calculated by Studier (79). Since the $S_{20,w}$ shows no concentration dependence under these conditions (79), the intrinsic sedimentation coefficient ($S^{\circ}_{20,w}$) for a polynucleotide can be assumed to be equivalent to $S_{20,w}$. The molecular weights were calculated using the following Studier formulae (79):

(a) neutral pH

$$S^{\circ}_{20,w} = 0.0882\ M^{0.346}$$

(b) alkaline pH

$$S^{\circ}_{20,w} = 0.0528\ M^{0.400}$$

where M = molecular weight.

Freifelder (87) has derived a different relationship between $S^{\circ}_{20,w}$ and molecular weight for neutral pH conditions:

$$S^{\circ}_{20,w} = 2.8 + 0.00834\ M^{0.479}$$

The molecular weight calculated from this relationship is different than the molecular weight determined by the Studier method. For example, an $S_{20,w}$ of 6 yields a molecular weight of 251,000 by the Freifelder formula, and a molecular weight of 200,000 by the Studier relationship. Both methods, however, are probably not reliable for low molecular weight DNAs since no low molecular weight standards exist. Since we are concerned more with relative, rather than absolute molecular weights, the Studier relationships are adequate.

N. Acrylamide Gel Electrophoresis

Gels (0.5 cm x 6.5 cm), prepared as described by Shapiro, Viñuela, and Maizel (88), consisted of either 5 or 10% acrylamide, the corresponding concentration of N,N'-methylene-bis-acrylamide, 0.05 M sodium phosphate, pH 7.2, and 0.1% SDS. Polymerization was catalyzed by N,N,N',N'-tetramethylene diamine and ammonium persulphate at final concentrations of 0.05% and 0.07%, respectively.

Protein samples were dialyzed overnight against 5 mM sodium phosphate, pH 7.2, and concentrated by evaporation. Protein samples (5-10 μg) were denatured by heating to 85° for 15 minutes in 10 mM dithiothreitol and 0.33% SDS. (This procedure was sufficient to lyse R17 phage, the source of the coat protein used as marker.) Samples were made 20% in glycerol, applied to the gels by layering beneath the electrophoresis buffer (0.1% SDS in 0.05 M sodium phosphate, pH 7.2), and electrophoresed at room temperature for 2 hours at 5 milliamps per gel. Gels were stained at 37° for 1.5 hours in 9.8% acetic acid, 45% methanol, and 0.24% Coomassie brilliant blue (89). Destaining was done in 7% acetic acid using a Canalco horizontal destainer. Densitometric tracings of stained gels were performed with a Gilford spectrophotometer linear transport system at 540 nm.

Molecular weights were determined from SDS-acrylamide gel electrophoresis by comparing the migration distances of the unknown proteins to those of proteins of known molecular weight.

CHAPTER III

PRODUCTION OF CLC DNA

I. Introduction

A certain fraction of the DNA synthesized *in vitro* by *E. coli* DNA polymerase I has covalent linkers between complementary sequences (CLC DNA). This has been shown for the copying of natural (61) and defined (62) DNAs. In this chapter, the occurrence of CLC structures in various DNAs, synthesized *in vitro* by *E. coli* DNA polymerase I, was determined by the fluorescence assay and, where possible, alkaline CsCl equilibrium density banding.

II. Results

A. *E. coli* DNA Synthesized *In vitro*

E. coli DNA was used as a template for a DNA polymerase I reaction. Portions were removed at various times and analyzed by the fluorescence method using the KE buffer system to determine the total DNA synthesized and its CLC content (Figure 8). The newly made polymer was 100% CLC, whereas the input template was 0% CLC.

B. Production of CLC DNA in Chemically Defined DNAs

Several defined DNAs were used as templates for DNA polymerase I. After isolation, the products were analyzed for CLC content by the fluorescence method and the ultracentrifugation method. Table IV summarizes the results. Polymers of the type polypyrimidine·polypurine have not been observed to produce CLC DNA.

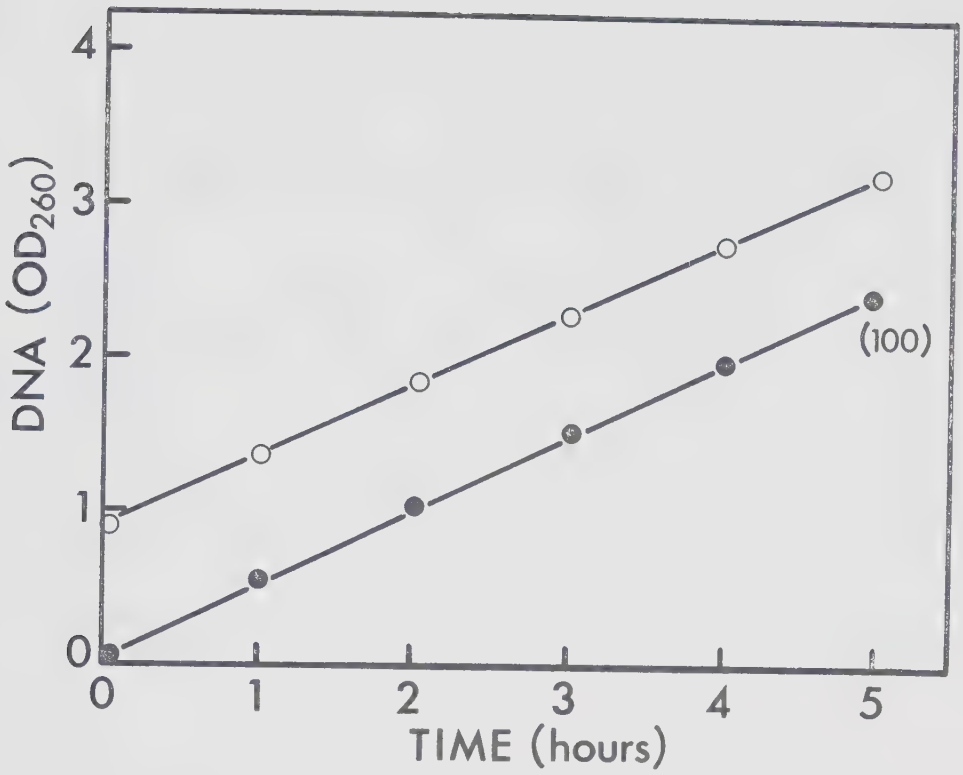


FIGURE 8. E. coli DNA Synthesis.
E. coli DNA, 0% CLC, at 1 OD_{260} was used as a template for DNA polymerase I under standard synthesizing conditions. Samples were analyzed at various times either directly or after heating and cooling in KE buffer. The number 100 represents the percentage of the newly made DNA which was CLC. O———O , total DNA; ●———● , CLC DNA.

TABLE IV

PRODUCTION OF CLC DNA IN VARIOUS DEFINED DNAs

Polymer	% CLC	CsCl Gradient
$d(T\text{-}G)_n \cdot d(C\text{-}A)_n$	25	Figure 6a
$d(T\text{-}T\text{-}G)_n \cdot d(C\text{-}A\text{-}A)_n$[a]	33	Figure 9
$d(T\text{-}C\text{-}C)_n \cdot d(G\text{-}G\text{-}A)_n$	2	Figure 10

[a] The template used was probably CLC in nature.

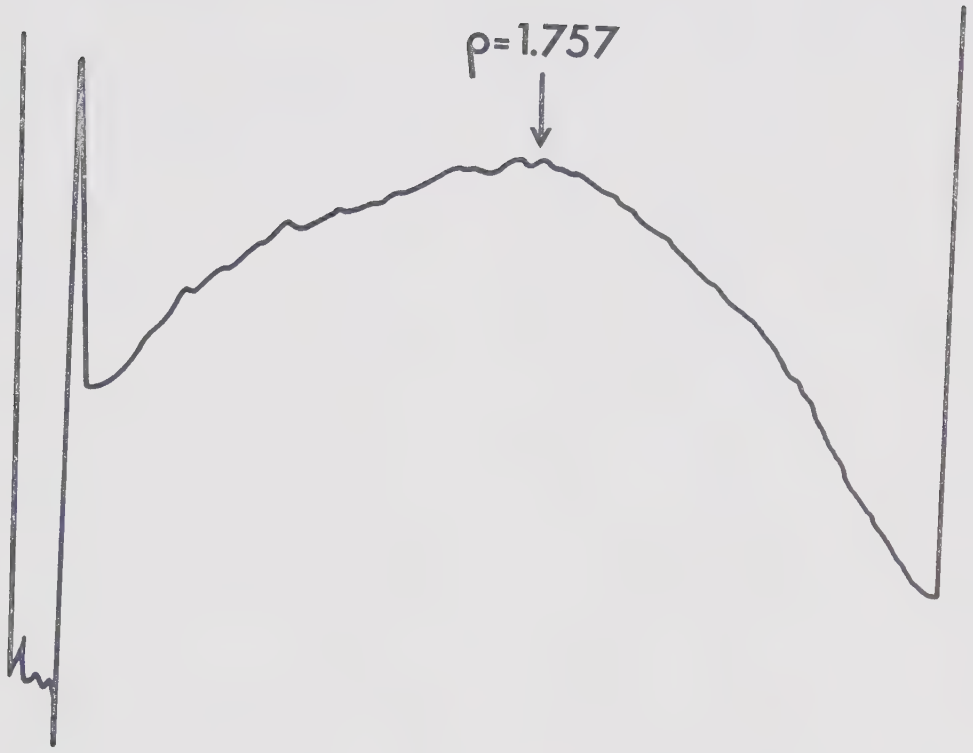
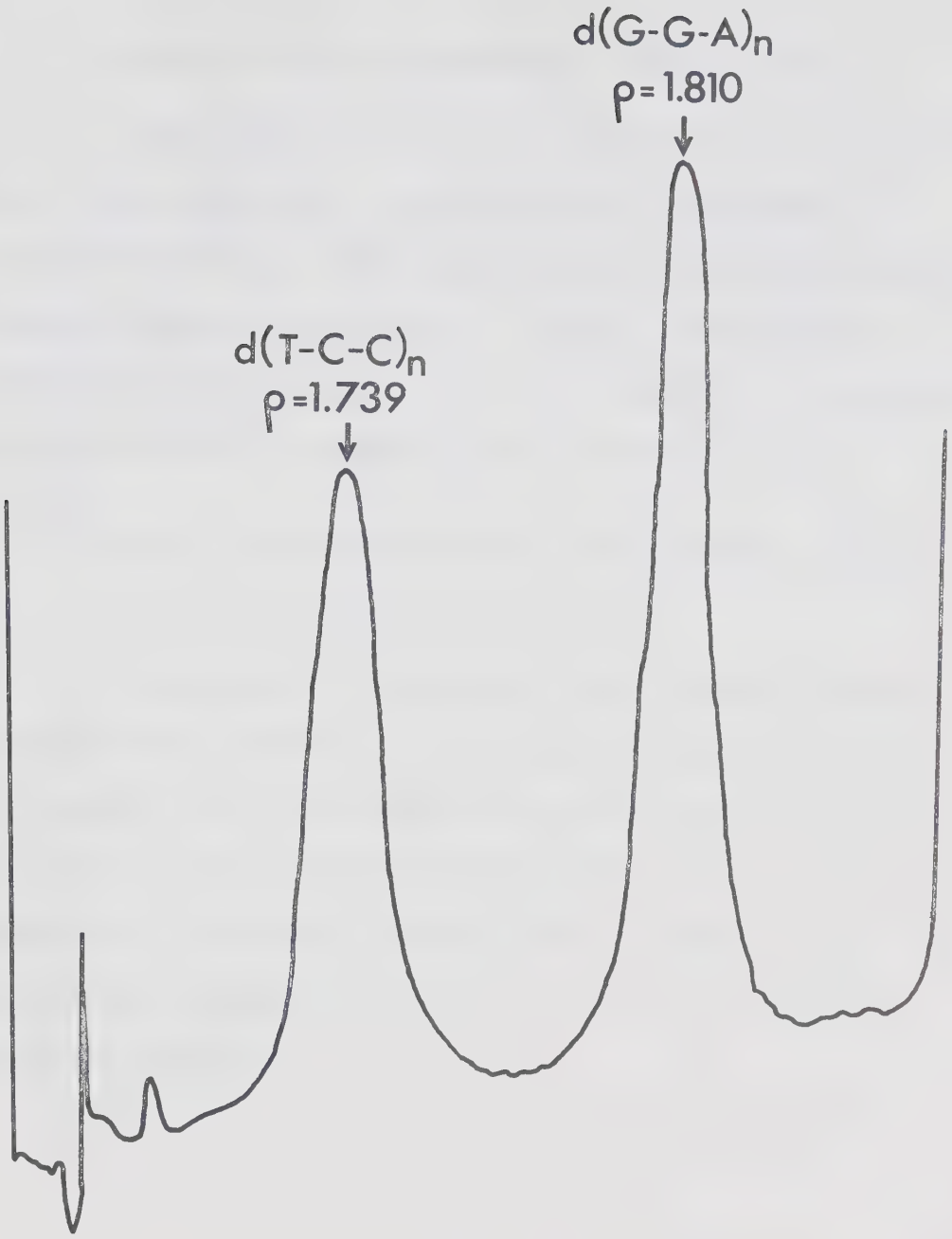


FIGURE 9. Analytical CsCl Ultracentrifugation of $d(T\text{-}T\text{-}G)_n \cdot d(C\text{-}A\text{-}A)_n$. The polymer was synthesized as indicated in Table IV, isolated by Agarose chromatography, and centrifuged to equilibrium in alkaline CsCl. Conditions of centrifugation were 48,000 rpm, at 20°, for 20 hours. The density was determined by the isoconcentration method (83).

FIGURE 10. Analytical CsCl Ultracentrifugation of $d(T\text{-}C\text{-}C)_n \cdot d(G\text{-}G\text{-}A)_n$.
The polymer was synthesized as indicated in Table IV, isolated by Agarose chromatography, and centrifuged to equilibrium in alkaline CsCl. Conditions of centrifugation were 48,000 rpm, at 20°, for 22 hours. Densities were determined by the isoconcentration method (83). On the basis of T and G content, $d(T\text{-}C\text{-}C)_n$ was assigned a density of 1.739 g/cm^3; $d(G\text{-}G\text{-}A)_n$, a density of 1.810 g/cm^3.


d(G-G-A)n
ρ= 1.810
d(T-C-C)n
ρ=1.739

C. Kinetics of CLC DNA Production in $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$

When $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ with separable strands was used as a template for DNA polymerase I, CLC structures did not appear for about 1 hour (Figure 11). After about 2-fold copying of the template had occurred (initial template was at 0.2 OD_{260}), CLC sequences appeared and increased rapidly to 25% of the total DNA. This polymer typically gave 20-25% CLC content by the fluorescence assay.

D. Effect of the Ratio of DNA to DNA Polymerase on the Production of CLC $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$

Reactions were carried out under standard conditions with a constant DNA polymerase concentration and varying levels of initial template. Total DNA and CLC DNA were determined at various times by the fluorescence assay (Table V). The production of CLC DNA was independent of the ratio of DNA to enzyme. Similar results were obtained using a constant template concentration but varying the polymerase concentration.

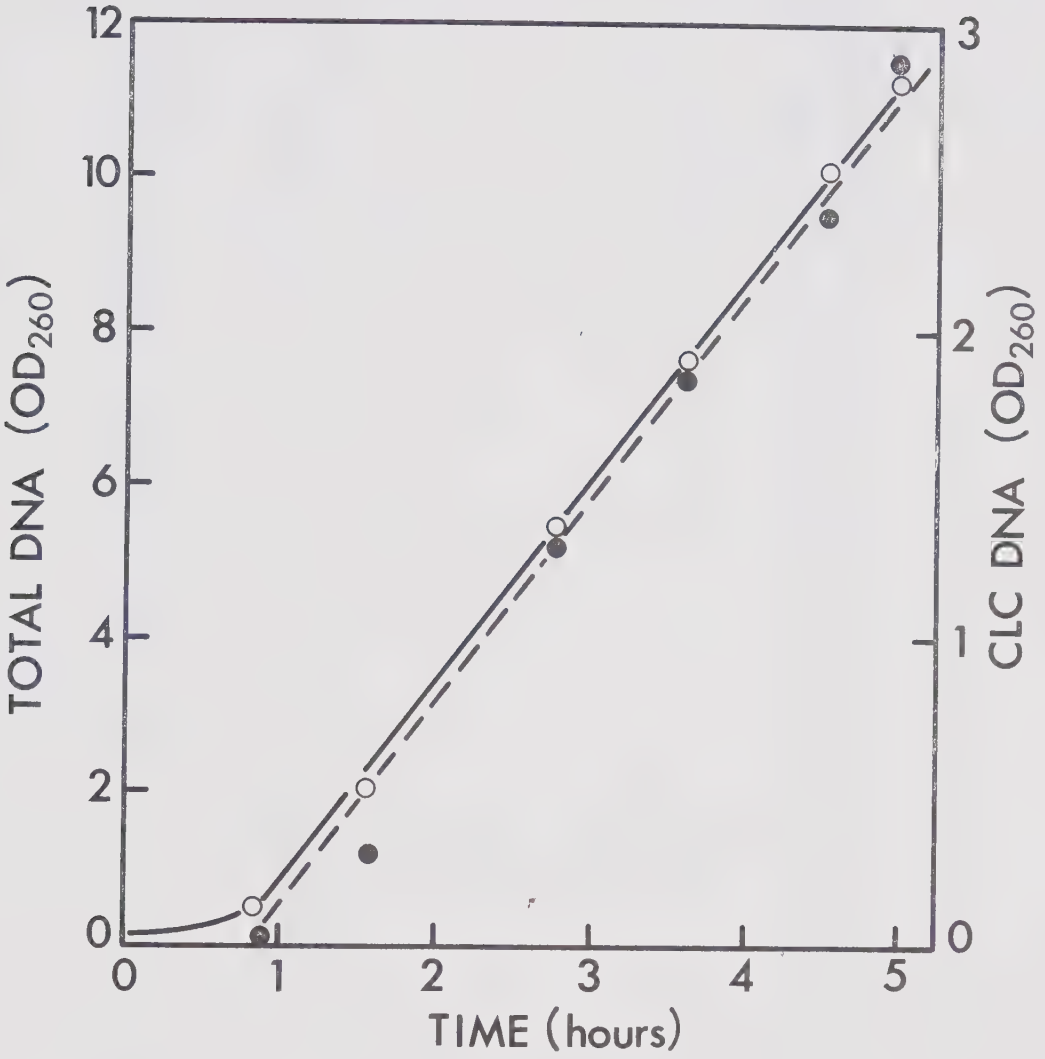


FIGURE 11. Kinetics of CLC $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$.
The polymer was synthesized under standard conditions with DNA polymerase I. At various times, total DNA synthesized and its CLC content were determined by the fluorescence method. O———O , total DNA; ●-----● , CLC DNA.

TABLE V

EFFECT OF THE RATIO OF DNA TO POLYMERASE ON THE PRODUCTION OF CLC $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$

Time (minutes)	0	30		45	
	DNA/enzyme[a]	DNA/enzyme[a]	% CLC[b]	DNA/enzyme[a]	% CLC[b]
	0.16	0.46	10	0.69	17.6
	0.31	0.84	11	1.22	17.2
	0.62	1.45	12	2.00	18.0
	1.60	2.68	12	3.32	17.0
	3.10	4.60	11	5.40	17.2
	6.20	7.90	10	8.30	17.3

[a] The ratios of DNA to enzyme are expressed in terms of the number of molecules present based on a molecular weight for $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ of 350,000, and a molecular weight for *E. coli* DNA polymerase I of 110,000.

[b] The fluorescence assay was used to determine the CLC DNA content.

III. Discussion

In vitro CLC DNA production is not peculiar to *E. coli* DNA polymerase I. *Micrococcus luteus* DNA polymerase (90), avian myeloblastosis virus DNA polymerase (91,92), T4 DNA polymerase (54), and calf thymus DNA polymerase (59) all synthesize some CLC DNA structures. The mechanism of CLC DNA formation probably involves either turning around (Figure 3b) (59), or a combination of strand switching (Figure 3a) (93) and turning around by the polymerase, as discussed in Chapter VI. *E. coli* DNA polymerase II (57) and polymerase III (58) are limited to repair synthesis *in vitro*. Since net-fold synthesis (more product than template) may be essential for CLC DNA production, these enzymes may not produce CLC DNA *in vitro*.

Natural DNAs synthesized with *E. coli* DNA polymerase I *in vitro* contain 100% CLC sequences. Synthesis of certain defined DNA, for example, $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ and $d(T\text{-}T\text{-}G)_n \cdot d(C\text{-}A\text{-}A)_n$ produces some CLC structures. The production of such structures is independent of the ratio of DNA to enzyme.

Polypyrimidine·polypurine DNAs, besides failing to produce CLC sequences during synthesis, are also synthesized at a slower rate than other DNA polymers. Synthesis can be stimulated by the addition of Fraction DIII (Chapter II). This stimulation cannot be mimicked by any combination of the following nucleases: pancreatic DNase I, endonuclease I, or exonuclease III (Coulter, M.B., Flintoff, W.F., and Paetkau, V.H.: unpublished observations). We have observed that DIII contains low molecular weight oligonucleotides which act as templates for DNA polymerase I (Coulter, M.B., Flintoff, W.F., and Paetkau, V.H.: unpublished observations). The products of this

synthesis are high molecular weight DNAs of the polypyrimidine·polypurine type, as characterized by their ability to act as templates for poly rG synthesis by E. coli RNA polymerase [by the method described by Paetkau et al. (94)]. It is possible that these low molecular weight oligomers are acting as primers when high molecular weight polypyrimidine·polypurine DNAs are used as templates.

Schandl (95) has reported that pulse labelled oligodeoxynucleotides from HeLa cells stimulate in vitro DNA synthesis catalyzed by E. coli DNA polymerase I. Since these oligomers are incorporated into the product, they are probably acting as primers. Similar oligomers have been isolated from pulse-labelled bacterial cells (96). Whether such oligomers are of the polypyrimidine·polypurine type has yet to be established.

CHAPTER IV

PURIFICATION AND PROPERTIES OF S FACTOR

I. Introduction

An earlier report (62) indicated the existence of a heat labile material in E. coli that prevented the accumulation of CLC sequences during the synthesis of $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$. This initial observation was made with Fraction IV DNA polymerase (76). Subsequent work indicated that Fraction DIII (Chapter II) contained a similar activity. Preliminary experiments indicated that this activity in DIII could not be mimicked by E. coli endonuclease I in the presence or absence of tRNA, by protamine sulfate, by ammonium sulfate, or by ionic strength (Coulter, M., Flintoff, W., Paetkau, V., Pulleyblank, D., and Morgan, A.R.: manuscript submitted). Unlike Fraction IV, this activity was partially heat stable in DIII, perhaps due to the presence of protamine sulfate used in its isolation.

A purification scheme was developed for S factor (strand separability factor) from Fraction IV. Fraction IV was used rather than DIII because of the presence in DIII of protamine sulfate and a poly-pyrimidine·polypurine type DNA, both of which were difficult to remove.

II. Methods

A. Nuclease Assays

Nuclease activity was assayed by the following methods.

(i) The loss of label from acid insoluble polynucleotide was determined in a reaction mixture containing 30 mM potassium phosphate, pH 7.4, 12 mM $MgCl_2$, 2 mM β-mercaptoethanol, either 0.52 OD_{260} $^3H\text{-}d(\overset{*}{T}\text{-}G)_n \cdot {}^{14}C\text{-}d(\overset{*}{C}\text{-}A)_n$ (120,000 cpm/OD_{260} unit), or 0.23 OD_{260} $^3H\text{-}d(\overset{*}{T}\text{-}G)_n$ (90,000 cpm/OD_{260} unit), or 0.23 OD_{260} $^{14}C\text{-}d(\overset{*}{C}\text{-}A)_n$ (77,000 cpm/OD_{260} unit), various S factor fractions, and, where indicated, 0.5 OD_{260} yeast tRNA. Incubations were at 37°. Portions were removed at various times and acid insoluble radioactivity determined. One nuclease unit was defined as one nmole of nucleotide released per minute at 37°. This calculation was based on a molar extinction coefficient of 6.5×10^3 for $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ (97) and 9×10^3 for the single-stranded DNAs.

(ii) The conversion of covalently closed circular (CCC) PM2 DNA to a relaxed molecule was determined by fluorescence. The reaction mix contained 40 mM potassium phosphate, pH 7.4, 8 mM $MgCl_2$, 1 OD_{260} CCC PM2 DNA, and 10 µg/ml Fraction 8 S factor. At various times, portions were diluted into KE buffer containing 0.5 µg/ml ethidium bromide and the fluorescence measured before and after heating. The CCC DNA renatures after heating, but nicked molecules do not. Any endonuclease activity would be indicated by a loss in fluorescence after heating since under these conditions single-stranded DNA does not fluoresce.

B. $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$ Synthesis

The presence of low molecular weight $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$ in S factor fractions was determined with a synthesis reaction using DNA polymerase I. The reaction mix contained 67 mM potassium phosphate,

pH 7.4, 6.7 mM $MgCl_2$, 2 mM β-mercaptoethanol, 0.2 mM dATP, 0.2 mM ^{3}H-TTP (specific activity 3000 cpm/nmole), 0.5 OD_{260} tRNA, 1.25 µg/ml Fraction 8 DNA polymerase I, and various levels of S factor fractions. Incubations were at 37°. At various times, portions were removed and acid insoluble radioactivity determined. The amount of $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$ synthesized was calculated from the radioactivity incorporated using a molecular extinction coefficient of 6.7×10^3 for $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$ (97).

C. Calibration of Sephadex G-75 Column

A mixture (5 mls) containing 0.1% Blue Dextran 2000, 30 OD_{260} units ATP, 1 mg ovalbumin, 1 mg chymotrypsinogen A, 1 mg sperm whale myoglobin and 0.5 mg cytochrome c was chromatographed on the Sephadex G-75 column (4.91 cm^2 x 95 cm) under identical conditions used to prepare S factor Fraction 6 (see text). The elution of each component was determined optically - OD_{280}: Blue Dextran, ovalbumin, chymotrypsinogen A; OD_{260}: ATP; OD_{430}: sperm whale myoglobin; OD_{410}: cytochrome c.

D. Purification of E. coli RNA Polymerase

RNA polymerase from E. coli B was prepared to Fraction 3 by the method of Burgess (98) and further purified as for Fraction III to VI by the method of Paetkau and Coy (74) omitting the 0.5 M Agarose step and substituting DEAE-Sephadex for QAE-Sephadex. The purified enzyme had a specific activity of about 10,000 units per mg of protein (one unit corresponds to the incorporation of 1 nmole of ^{14}C-CMP per hour under the assay conditions described by Chamberlin and Berg (75) except that calf thymus DNA was used as template, instead of salmon sperm DNA).

E. Transcription of T4 DNA

The transcription was done at 37° in an incubation mixture containing 0.04 M Tris-Cl, pH 8.0; 4 mM $MgCl_2$; 0.8 mM $MnCl_2$; 10 mM β-mercaptoethanol; 40 mM KCl; 0.4 mM ^{3}H-CTP, specific activity 100 cpm/pmole; 0.4 mM of each of the other rNTPs; 0.016 or 0.04 OD_{260} T4 DNA, 2 µg/ml Fraction VI RNA polymerase (sigma containing); and various levels of Fraction 8 S factor. Portions were removed at various times and RNA synthesis followed by the incorporation of label into acid-insoluble polynucleotide (74).

III. Results

Unless otherwise indicated, all operations were carried out at 0°-4° and centrifugations were at 10,000 x g for 10 minutes. A typical purification procedure is summarized in Table VI.

A. Steps 1-4

The initial steps - grinding, streptomycin precipitation, autolysis, and ammonium sulfate fractionation - corresponded to Steps 1 to 4 of the DNA polymerase I purification from E. coli B (76) except that the ammonium sulfate precipitate was dissolved to yield a protein concentration of about 40 mg/ml.

B. Step 5: DEAE-Cellulose Chromatography I

This treatment removed high molecular weight nucleotidic material without the adsorption of S factor. One ml of 1 M potassium phosphate, pH 6.5, was added to 20 mls of Fraction 4 (material from 1 lb. of cells) to lower the pH and to increase the phosphate concentration to 0.07. The sample was centrifuged and applied under moderate pressure

TABLE VI

PURIFICATION OF S FACTOR[a]

	Total Protein mg	Total Units	Specific Activity units/mg	Yield[d] %
4. Ammonium sulfate	740[b]	12,000	16.2	100
5. DEAE-cellulose I	570[b]	10,800	18.9	90
6. Sephadex G-75	3.2[c]	7,500	2,300	62.5
7. Urea-LiCl	3.1[c]	3,900	1,280	32.5
8. DEAE-cellulose II	0.38[c]	720	1,880	6[e]

[a] from 1 lb. of E. coli B cells

[b] determined by Biuret method

[c] determined by OD_{280}

[d] based on units, see page 28

[e] 50% of the enzyme preparation was subjected to Step 8. The values in the table are corrected to 100%.

to a DEAE-cellulose column (0.63 cm^2 x 7 cm) equilibrated with Buffer A (0.2 M potassium phosphate, pH 6.5, 10 mM β-mercaptoethanol, and 1 mM EDTA). The flow rate was 0.3-0.4 ml/minute. The protein wash-through was collected as Fraction 5.

Most of the S factor activity in Fraction 5 was completely excluded from Sephadex G-75. Autolysis, as described below, quantitatively converted it to a partially included form. The autolysis was possible only after low molecular weight inhibitory components had been removed and Mg^{++} added, as follows.

(i) Salt Exchange of Fraction 5

This treatment separated the protein from the low molecular weight nucleotidic material and salts. Fraction 5, 26 mls, was applied to a Sephadex G-25 column (4.91 cm^2 x 38 cm) equilibrated with Buffer A. The flow rate was 0.5 ml/minute and 12.6 ml fractions were collected (Figure 12). The excluded protein peak had an OD_{280} to OD_{260} ratio of 1.49, compared to 0.68 for Fraction 5.

(ii) Concentration

The excluded Sephadex G-25 protein peak, 38 mls, was concentrated to 6 mls using an Amicon ultrafiltration cell (Model 52) with a PM10 membrane at an operating pressure of 45 lb./in^2 of nitrogen.

(iii) Autolysis

This treatment quantitatively dissociated S factor from higher molecular weight components. The concentrated sample was centrifuged and magnesium chloride (final concentration 4 mM) added. Incubation was at room temperature. The extent of autolysis was measured by the acid solubilization of 0.52 OD_{260} $^{3}H\text{-}d(\overset{*}{T}\text{-}G)_n \cdot {}^{14}C\text{-}d(\overset{*}{C}\text{-}A)_n$

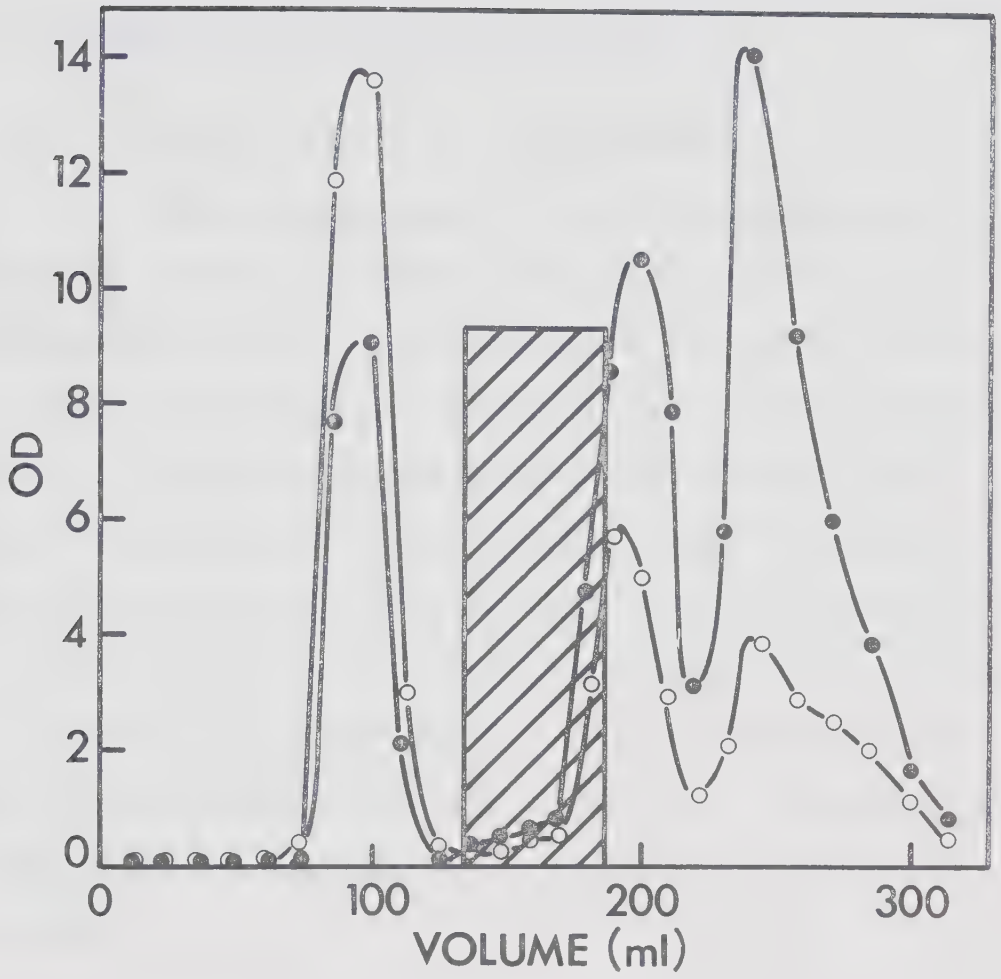


FIGURE 12. Salt Exchange of Fraction 5 S Factor.
Fraction 5, 26 mls, was applied to a Sephadex G-25 column (4.91 cm^2 x 38 cm) equilibrated with 0.2 M potassium phosphate, pH 6.5, 10 mM β-mercaptoethanol, and 1 mM EDTA. Fractions were 12.6 mls. Salt elution was monitored refractometrically. O———O, OD_{280}; ●———●, OD_{260}; ▨, salt elution.

(120,000 cpm/OD_{260} unit) added to a portion of the sample. The autolysis was stopped after 75 minutes when the polymer had been completely degraded. The time for total degradation varied between 60 and 120 minutes for different preparations of concentrated Fraction 5.

C. Step 6: Sephadex G-75 Chromatography

The autolyzed sample, 6 mls, was centrifuged and applied under gravity to a Sephadex G-75 column (4.91 cm^2 x 95 cm) equilibrated with Buffer A. After the sample had entered the resin, the flow rate was maintained at 15 mls/hour with an LKB peristaltic pump. Fractions of 5.2 mls were collected. The S factor activity was separated from the bulk of the protein and eluted at 50% bed volume (Figure 13) which corresponded to a molecular weight of 26,000. The fractions containing S factor activity were pooled (25 mls) and passed over a Sephadex G-25 column (4.91 cm^2 x 38 cm) equilibrated with Buffer B (10 mM Tris-Cl, pH 8.0, and 0.1 mM EDTA). The excluded protein was concentrated by lyophilization and dissolved in 3 mls of water (Fraction 6).

D. Properties of Fraction 6

(i) Nuclease content

Most of the nuclease in Fraction 6 was active on a double-stranded template (Table VII). This nuclease activity varied from preparation to preparation and was 50-60% endonuclease I as indicated by its tRNA sensitivity (78). At the level of Fraction 6 required to reduce the CLC DNA content of $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ by 50%, it would have

FIGURE 13. Sephadex G-75 Chromatography.
The autolyzed sample (6 mls Fraction 5) was applied to a Sephadex G-75 column (4.91 cm^2 x 95 cm) equilibrated with 0.2 M potassium phosphate, pH 6.5, 10 mM β-mercaptoethanol, and 1 mM EDTA. The flow rate was 15 mls/hour and 5.2 ml fractions were collected. The S factor activity was determined under standard conditions using the fluorescence assay on $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ polymers. A 50% decrease in the CLC content of $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ was defined as one unit of S factor activity. The elution (V_o or V_e) of Blue Dextran 2000, ovalbumin, chymotrypsinogen A, sperm whale myoglobin, cytochrome c, and ATP was determined separately. ○———○ , OD_{280}; ●———● , OD_{260}; △———△ , S factor activity, units/ml.

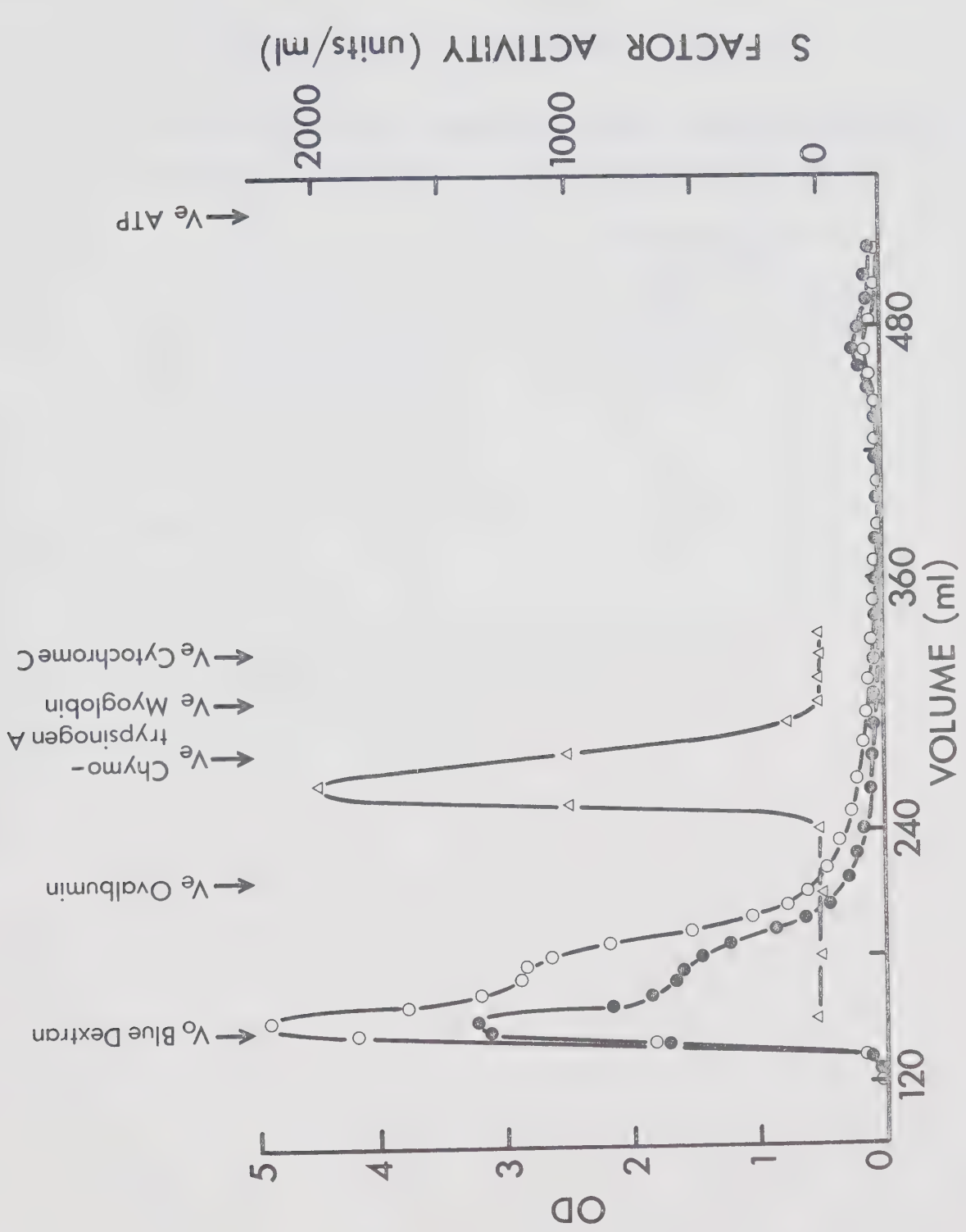

OD
VOLUME (ml)
S FACTOR ACTIVITY (units/ml)
Vo Blue Dextran
Ve Ovalbumin
Ve Chymo-
trypsinogen A
Ve Myoglobin
Ve Cytochrome C
Ve ATP

TABLE VII

NUCLEASE ACTIVITY OF FRACTION 6 S FACTOR

Nuclease assays were carried out under standard conditions using the acid solubilization of labelled polynucleotide.

Template	Nuclease Activity units/ml[a]	
	- tRNA	+ tRNA
$^{3}H\text{-}d(\overset{*}{T}\text{-}G)_n \cdot {}^{14}C\text{-}d(\overset{*}{C}\text{-}A)_n$	39.5	17.0
$^{3}H\text{-}d(\overset{*}{T}\text{-}G)_n$	2.6	1.1
$^{14}C\text{-}d(\overset{*}{C}\text{-}A)_n$	2.3	1.4

[a] One nuclease unit was defined as one nmole of nucleotide released/minute at 37°.

taken the tRNA insensitive nuclease 20-50 hours to completely degrade the input template.

(ii) $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$ Content

Unlike Fraction 5 S factor, Fraction 6 showed an increase in the CLC content of product at intermediate levels (Figure 14). The decrease in the ratio of ^{14}C-dC to ^{3}H-T incorporation (Inset, Figure 14) suggested that the increase in CLC was due to $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$ production. The following observations (Table VIII) supported this: (a) TTP incorporation was dependent upon dATP, (b) ^{14}dCTP was not incorporated, and (c) the product synthesized was of 100% CLC content. The kinetics of synthesis suggested that low molecular weight material was the template (Figure 15).

Incubation with magnesium chloride, or with exonuclease III, or adsorption and elution from DEAE-cellulose did not remove this $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$ template activity from S factor.

E. Step 7: Urea-Lithium Chloride Treatment

This treatment removed the $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$ template activity. The procedure was a modification of that used by Traub and Nomura to dissociate E. coli ribosomal proteins (99). To 1 ml of Fraction 6, 1 ml of an urea-lithium chloride solution (8 M in each component) and β-mercaptoethanol (final concentration 6 mM) were added. The sample was incubated in an ice slurry for 36 hours. The solution was centrifuged and applied under gravity to a Sephadex G-50 column (0.635 cm^2 x 55 cm) equilibrated with Buffer C (4 M urea, 4 M LiCl, 10 mM Tris-Cl, pH 7.5, and 6 mM β-mercaptoethanol). After the sample had entered the resin, the flow rate was maintained at 2.3 mls/hour

FIGURE 14. Titration Curves of S Factor Activity in Fraction 5 and Fraction 6.

The S factor activity in Fractions 5 and 6 was determined by the fluorescence assay on $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$. The polymers were synthesized with ^{3}H-TTP (specific activity 3000 cpm/nmole) and ^{14}C-dCTP (specific activity 1710 cpm/nmole). At the end of 5 hours, acid insoluble radioactivity and the CLC DNA content (fluorescence assay) were determined. ●———●, Fraction 5; ○———○, Fraction 6.

The ratios of ^{14}C-dC to ^{3}H-T incorporated in the polymers synthesized with various levels of Fraction 6 are shown in the Inset.

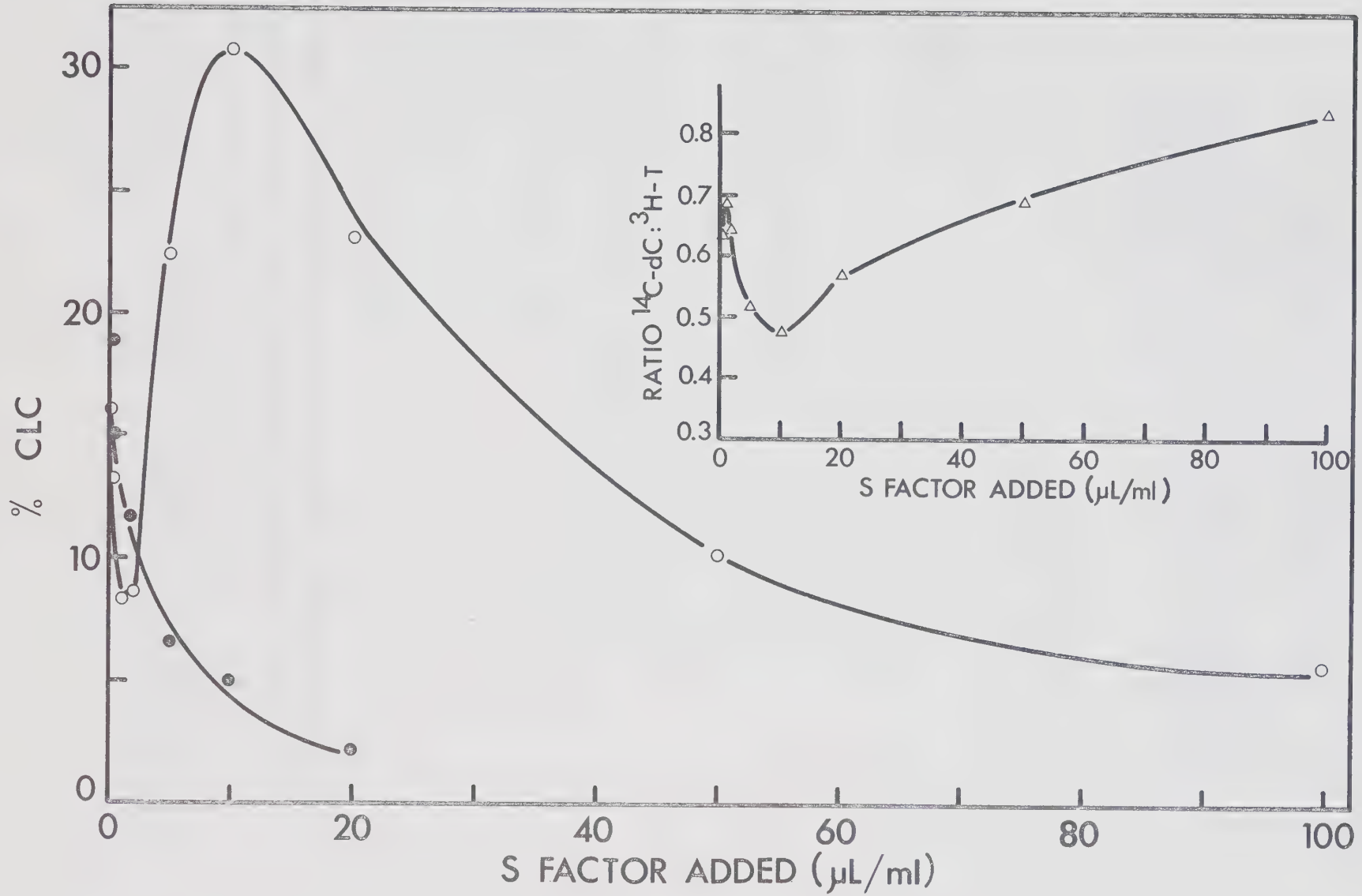

% CLC
30
20
10
0
0
20
40
60
80
100
S FACTOR ADDED (µL/ml)
RATIO ^{14}C-dC : ^{3}H-T
0.8
0.7
0.6
0.5
0.4
0.3
0
20
40
60
80
100
S FACTOR ADDED (µL/ml)

TABLE VIII

TEMPLATE ACTIVITY OF FRACTION 6 S FACTOR

Synthesis was done under the $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$ synthesizing conditions with either ^{3}H-TTP (specific activity 3000 cpm/nmole) or ^{14}C-dCTP (specific activity 1710 cpm/nmole) present.

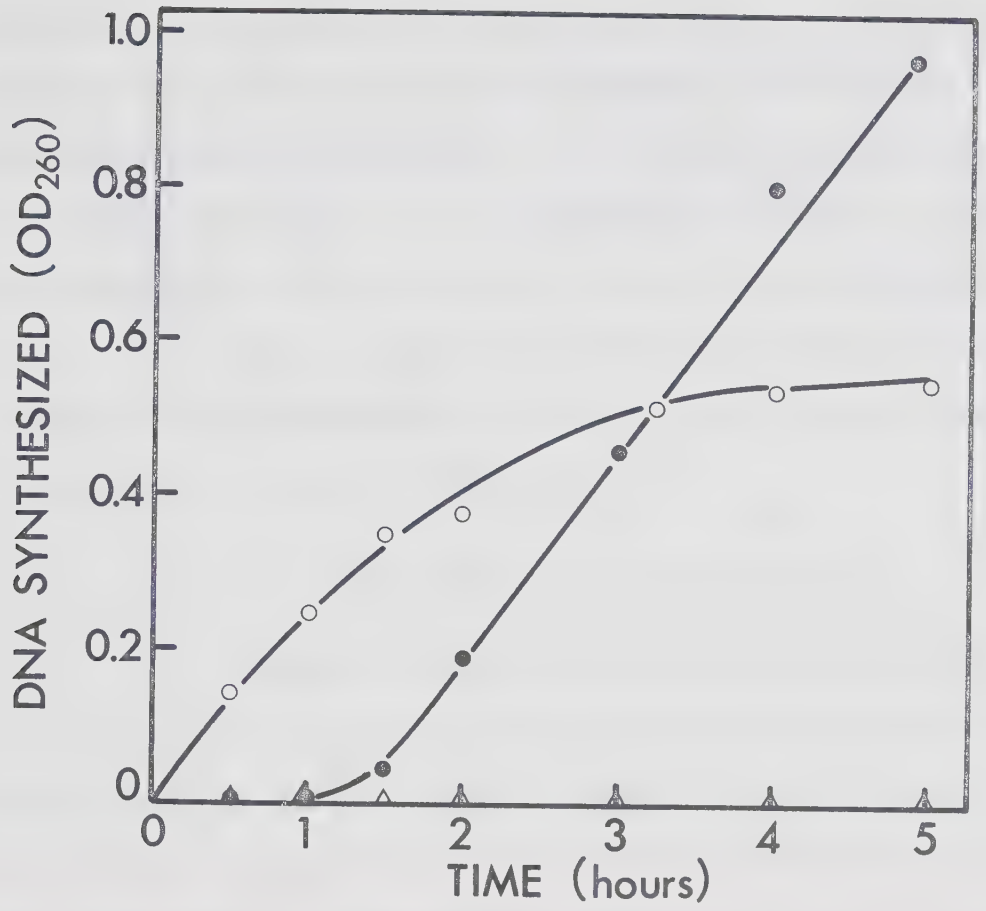
Conditions	DNA Synthesized OD_{260} at 5 Hours[a]	% CLC DNA at 6 Hours[b]
DNA polymerase I		
+ ^{3}H-TTP, dATP	0	-
DNA polymerase I + 0.2 OD_{260} $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$		
+ ^{3}H-TTP, dATP	0.55	97
DNA polymerase I + 20 µg/ml Fraction 6 S Factor		
+ ^{3}H-TTP, dATP	0.97	104
+ ^{3}H-TTP, dATP, dGTP, dCTP	0.69	97
+ ^{3}H-TTP	0	-
+ ^{3}H-TTP, dCTP	0	-
+ ^{3}H-TTP, dGTP	0	-
+ ^{14}C-dCTP, dGTP, dATP, TTP	0	-

[a]The amount of DNA synthesized was calculated from radioactive incorporation using a molar extinction coefficient of 6.7×10^3 for $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$ (97).

[b]The CLC content was determined by the fluorescence assay. By this assay, 0.02 OD_{260} of the defined polymers $d(A)_n \cdot d(T)_n$ and $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$ gave 2% and 95% CLC DNA content, respectively.

FIGURE 15. Kinetics of Putative $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$ Production from Fraction 6 S Factor.

Synthesis was carried out under the $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$ synthesizing conditions with ^{3}H-TTP (specific activity 3000 cpm/nmole) present. Portions were removed at various times and acid insoluble radioactivity determined. The amount of $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$ synthesized was determined as indicated in Table VIII. △———△ , DNA polymerase I; ○———○ , DNA polymerase I plus 0.2 OD_{260} $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$; ●———● , DNA polymerase I plus 20 µg/ml Fraction 6 S factor.


DNA SYNTHESIZED (OD_{260})
1.0
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
TIME (hours)

with an LKB peristaltic pump. Fractions of 0.9 ml were collected. The excluded peak, 5.4 mls, was dialyzed for 15 hours against 500 mls of Buffer D (6 M urea, 2 M LiCl, 10 mM Tris-Cl, pH 7.5, and 6 mM β-mercaptoethanol). Over a period of 48 hours this buffer was diluted by pumping Buffer E (0.5 M KCl and 5 mM sodium phosphate, pH 7.2) into the dialysis chamber at 40 mls/hour. The protein was passed over a Sephadex G-25 column (1.33 cm^2 x 45 cm) equilibrated with Buffer B, concentrated by lyophilization, and dissolved in 1.5 mls of water (Fraction 7).

Although there was a 50% loss of S factor activity, the increase in CLC characteristic of $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$ synthesis in Fraction 6 was absent in Fraction 7 (Figure 16).

F. Step 8: DEAE-Cellulose Chromatography II

Residual nuclease activity was removed by DEAE-cellulose chromatography. One ml of Fraction 7 was diluted 5-fold with 50 mM Tris-Cl, pH 8.0, and applied under gravity to a DEAE-cellulose column (0.18 cm^2 x 7 cm) equilibrated with Buffer F (50 mM Tris-Cl, pH 8.0, and 10% glycerol). The column was washed with 2 mls of Buffer F, and the protein eluted with a 30 ml linear gradient (15 mls of Buffer F 0.03 M in NaCl and 15 mls of Buffer F 0.45 M in NaCl. The flow rate was maintained at 7.8 mls/hour with an LKB peristaltic pump. Fractions of 1.3 mls were collected. The S factor eluted at a mean NaCl concentration of 0.15 M (Figure 17). Fractions containing S factor activity were pooled (6 mls), passed over a Sephadex G-25 column (1.33 cm^2 x 45 cm) equilibrated with Buffer B, concentrated by lyophilization, and dissolved in 1 ml of water (Fraction 8).

FIGURE 16. Titration Curves of S Factor Activity in Fraction 6, Fraction 7, and Fraction 8.

The S factor activity in Fractions 6, 7 and 8 was determined by the fluorescence assay on $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$. Synthesis was for 5 hours. O———O , Fraction 6; ●———● , Fraction 7; △———△ , Fraction 8.

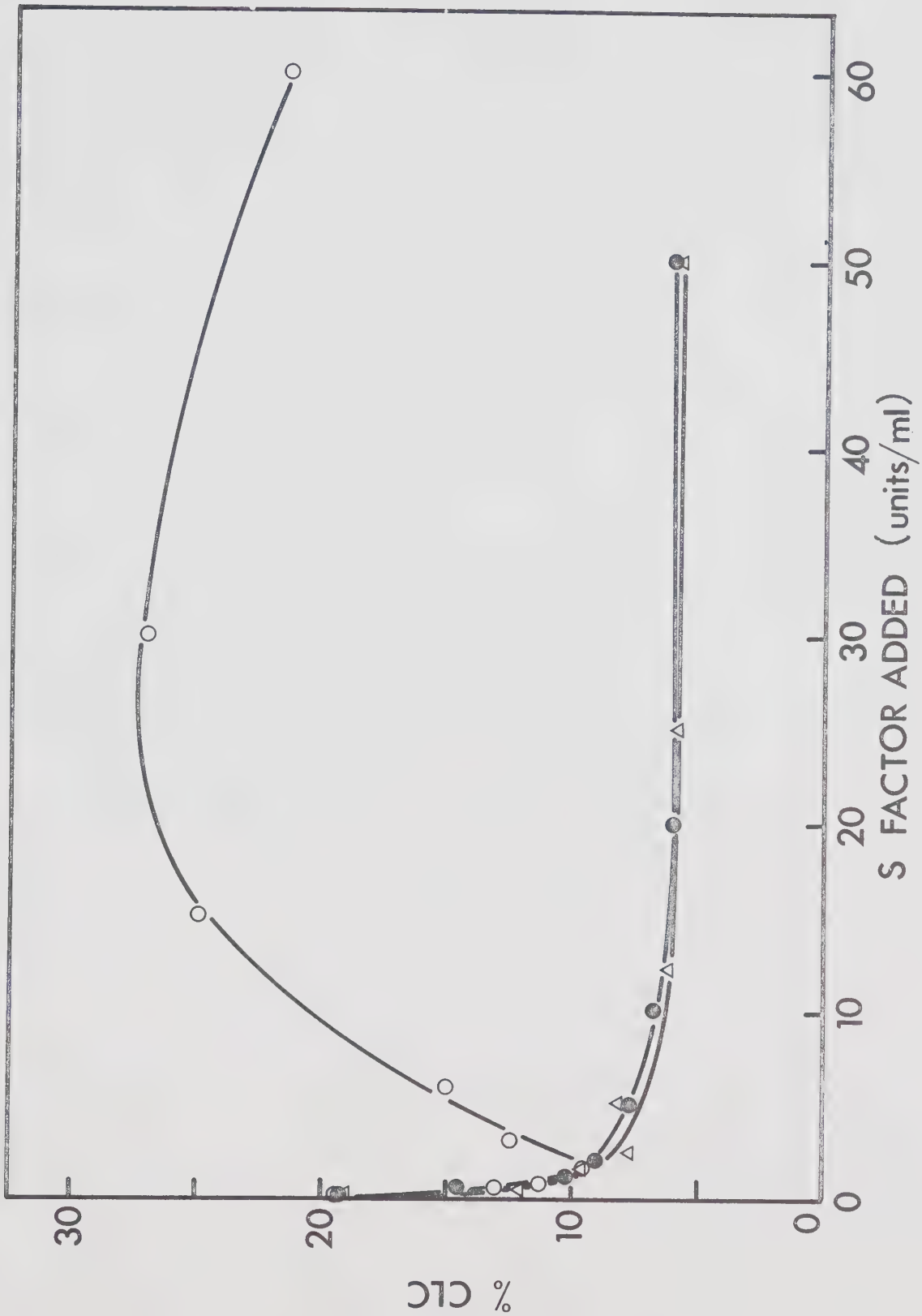
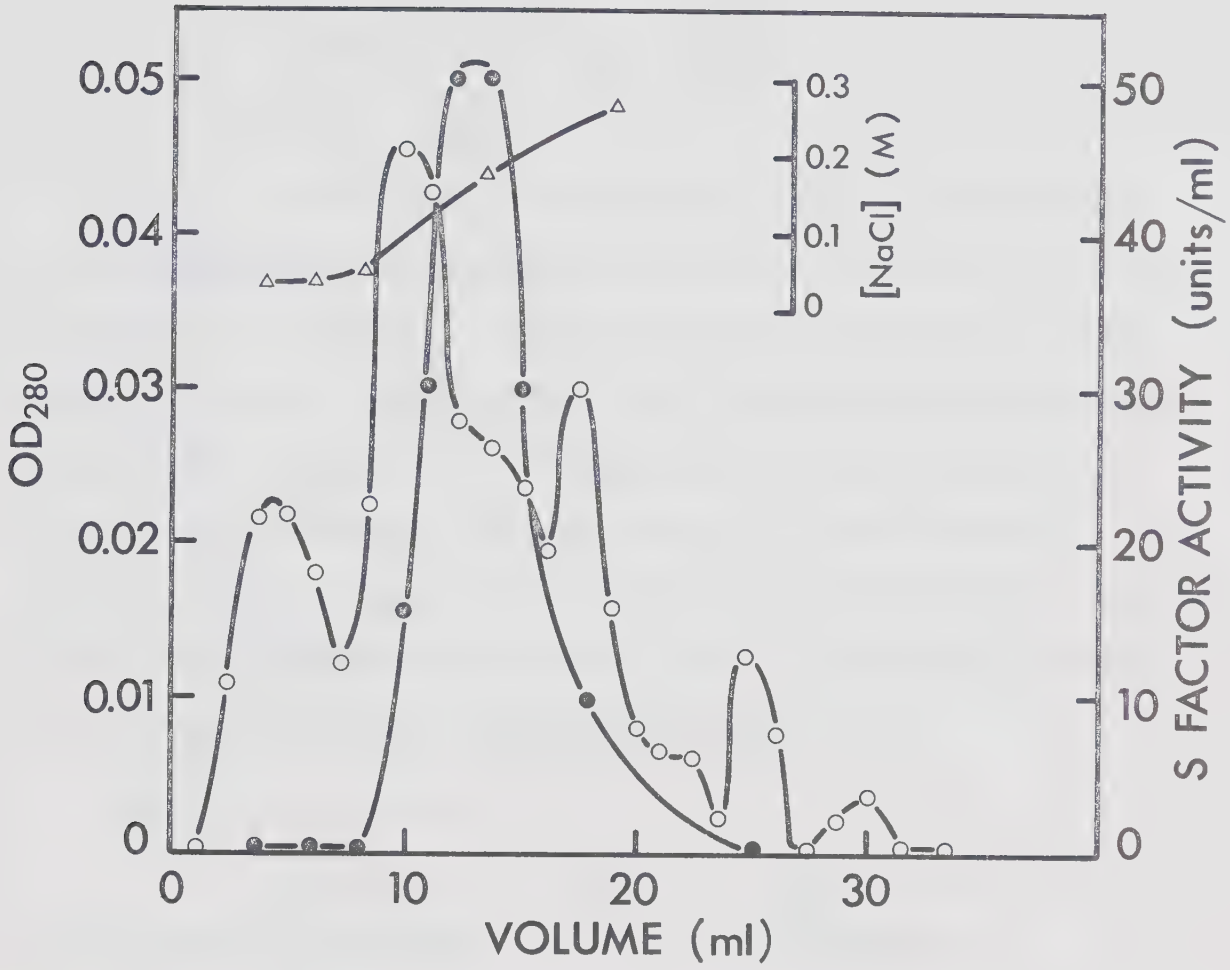

% CLC
30
20
10
0
0
10
20
30
40
50
60
S FACTOR ADDED (units/ml)

FIGURE 17. DEAE-Cellulose Chromatography II.

Fraction 7 S factor was treated as indicated in the text. The fluorescence assay on $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ was used to determine S factor activity. Sodium chloride concentrations were determined refractometrically. ○———○, OD_{280}; ●———●, S factor activity, units/ml; △———△, NaCl molarity.


0.05
0.04
0.03
0.02
0.01
0
OD280
0.3
0.2
0.1
0
[NaCl] (M)
50
40
30
20
10
0
S FACTOR ACTIVITY (units/ml)
0
10
20
30
VOLUME (ml)

Fraction 8 had an activity titration curve similar to Fraction 7 (Figure 16). This final chromatography, however, resulted in an 80% loss of activity. Fraction 8 has been stored on ice for 3 months with no loss of activity.

G. Properties of Fraction 8 S Factor

(i) Nuclease Content

With 10 µg/ml of Fraction 8, there was no detectable acid solubilization of radioactivity in 10 hours with either $^{3}H\text{-}d(\overset{*}{T}\text{-}G)_n$, $^{14}C\text{-}d(\overset{*}{G}\text{-}A)_n$ or $^{3}H\text{-}d(\overset{*}{T}\text{-}G)_n$. With the same level of Fraction 8, less than 10% of the PM2 DNA molecules (1 OD_{260}) were nicked in 20 hours of incubation as determined by the fluorescence nuclease assay. These upper limits of nuclease activity indicate that during polymer synthesis less than 1 nmole of nucleotide would be removed by exonuclease activity for every 400 nmoles incorporated, and less than 1 molecule in 4×10^4 would be nicked by endonuclease activity.

(ii) Heat Stability

When heated for 7 minutes at various temperatures, Fraction 8 lost 50% of its activity between 45° and 50° (Figure 18).

(iii) Analysis in SDS-Acrylamide Gel Electrophoresis

Densitometric tracings of stained gels, 10% in acrylamide, showed that 90-95% of the protein present in 4 µg of Fraction 8 migrated as a single band in SDS-acrylamide gel electrophoresis (Figure 19b). A similar gel pattern was obtained with 30 µg of Fraction 8. Fraction 7 contained 4 distinct protein bands (Figure 19a). The band with the greatest mobility corresponded to the band in

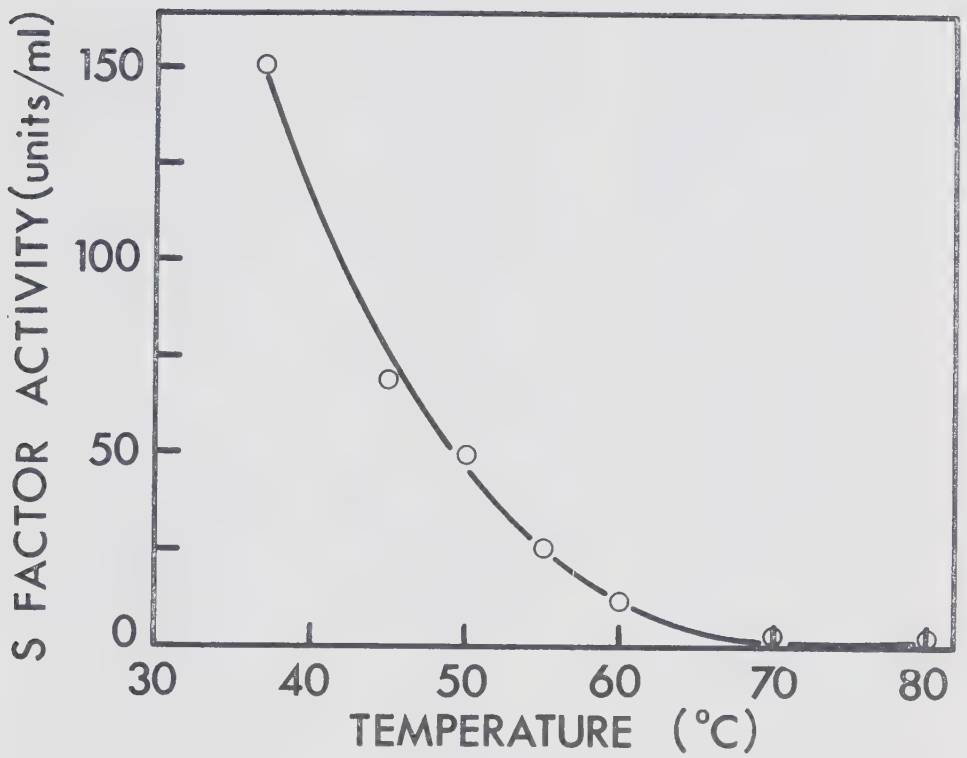


FIGURE 18. Heat Stability of Fraction 8 S Factor.
Fraction 8 S factor was heated to the indicated temperatures for 7 minutes, centrifuged, and assayed for residual S factor activity. Standard assay conditions were used and CLC content of the $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ polymers was determined by the fluorescence method.

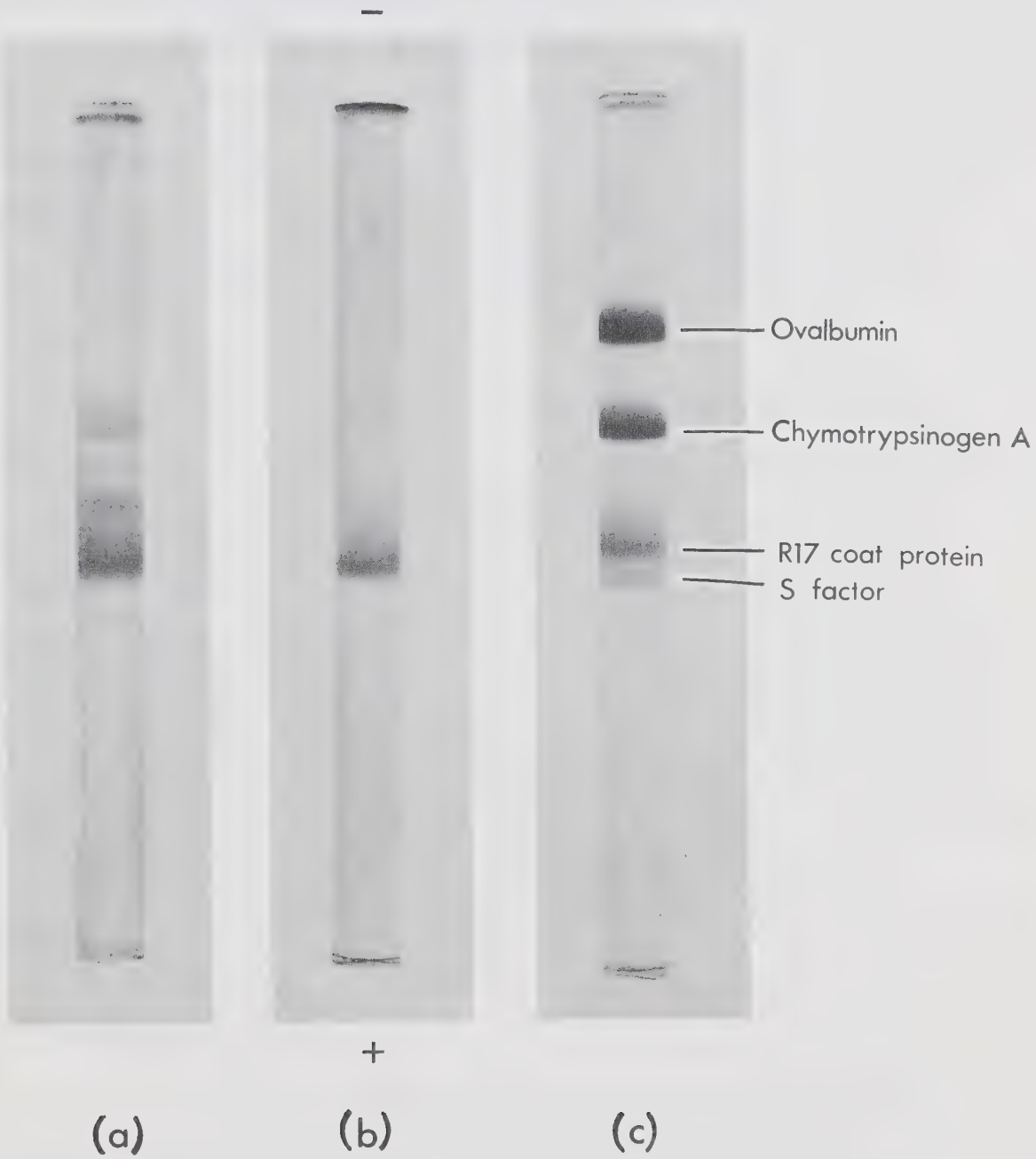
FIGURE 19. SDS-Acrylamide Gel Electrophoresis of S Factor and Marker Proteins.

Gels were 10% in acrylamide. Conditions of sample preparation and electrophoresis were as given in Chapter II.

(a) 10 μg of Fraction 7 S factor.

(b) 4 μg of Fraction 8 S factor.

(c) 7 μg of ovalbumin, 7 μg of chymotrypsinogen A, ∿ 4 μg of R17 coat protein (obtained from the lysis of R17 phage particles during the sample preparation), and 3 μg of Fraction 8 S factor.


−
Ovalbumin
Chymotrypsinogen A
R17 coat protein
S factor
+
(a)
(b)
(c)

Fraction 8. In some Fraction 7 preparations, this particular band was not the dominant species.

(iv) Molecular Weight Determination from SDS-Acrylamide Gel Electrophoresis

Fraction 8 S factor migrated slightly ahead of R17 coat protein (Figure 19c). By comparing the migration distance of S factor to the migration distances of proteins of known molecular weight, S factor was assigned a molecular weight of 11,500 (Figure 20).

(v) Effect on Transcription

Experiments were performed with ratios of Fraction 8 S factor to DNA varying between 1:1 and 20:1 (on a weight basis) to determine the effect of S factor on the transcription of T4 DNA by E. coli RNA polymerase. At all sampling times, 10, 20, 40, and 60 minutes, there was less than 10% difference between the amount of RNA synthesized in the absence or presence of S factor regardless of the ratio of S factor to DNA. In all cases, an amount of RNA equivalent to 70-80% of the DNA content was synthesized in 60 minutes. The S factor, thus, differs from the known low molecular weight transcription-stimulating proteins of E. coli (100,101).

IV. Discussion

The purification of S protein was complicated by the presence of nucleases and low molecular weight oligo d(A-T).

During the initial S factor purification steps, nuclease activities other than endonuclease I interfered with the assay system for S factor. These nucleases decreased the apparent CLC content of

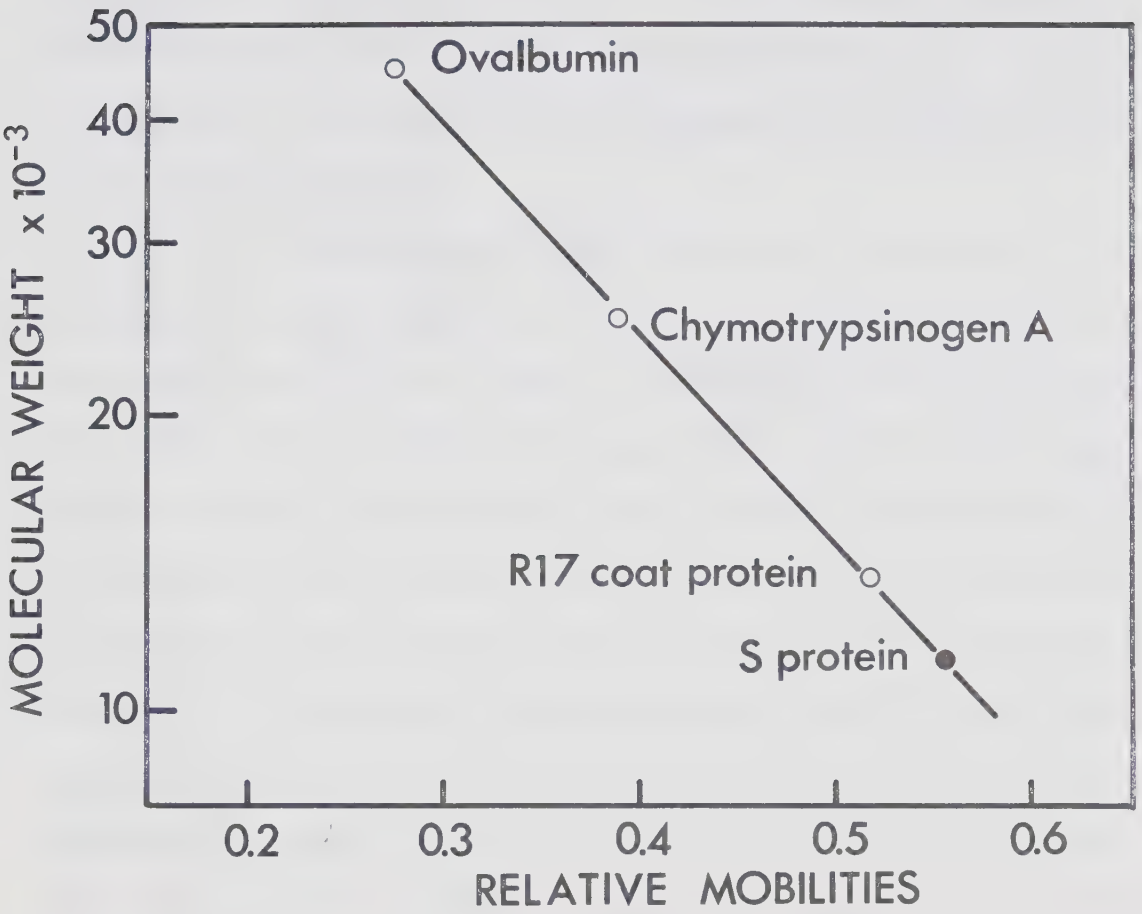


FIGURE 20. Molecular Weight Determination from SDS-Acrylamide Gel Electrophoresis.
The relative mobilities were calculated by dividing the distance the protein band migrated by the gel length. The molecular weights used for the marker proteins were: 45,000 for ovalbumin, 25,000 for chymotrypsinogen A, and 13,750 for R17 coat protein.

$d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ as measured by the fluorescence assay, and in some cases completely inhibited the synthetic reaction. The bulk of this nuclease was removed by Sephadex G-75 chromatography (Fraction 6). Residual activity was removed by subsequent DEAE-cellulose chromatography (Fraction 8). Fraction 8 S factor contained no detectable endonuclease or exonuclease activity.

Low molecular weight oligo d(A-T) was difficult to remove because of its association with S factor. The small size of the oligonucleotide was likely the result of degradation during the autolysis in Fraction 5. If this autolysis treatment was omitted, most of the S factor activity eluted in the excluded volume from the Sephadex G-75 column (Fraction 6). The observation that Fraction 6 S factor contained a template for $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$ synthesis suggests that E. coli DNA contains d(A-T) sequences. The binding of S factor to this oligonucleotide appears to be sequence specific since only $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$ was synthesized; however, binding to ends of the low molecular weight material may also be involved. During the synthesis of polymers, S factor probably dissociated from the oligo d(A-T). In cases where sufficient nuclease was present (Fraction 5, and high levels of Fraction 6), this "unmasked" polymer was probably degraded. In the absence of sufficient nuclease (intermediate levels of Fraction 6), it was used as template (Figure 16). This low molecular weight oligo d(A-T) was removed by an urea-lithium chloride treatment (Fraction 7). Whether a similar effect could have been achieved with high salt alone is not known. The urea may be essential to keep the protein soluble at high salt concentrations.

The loss of activity in going from Fraction 7 to Fraction 8 may be due to low protein concentrations. If the treatments are done in the reverse order, similar losses occur only during the final step (urea-lithium chloride treatment).

Both DNA polymerase I and S factor can be obtained from this purification scheme. The excluded protein peak from the Sephadex G-75 column can be treated as Fraction 5 in the standard purification procedure for DNA polymerase I (71).

The elution of S factor from the Sephadex G-75 column at a position corresponding to a molecular weight of 26,000 and its molecular weight as determined from SDS-acrylamide gel electrophoresis (11,500) suggest that S factor may exist as a dimer. In every preparation S factor eluted at the same position on the Sephadex G-75 column even though oligo d(A-T) was present, probably in different amounts. The S factor eluted at the same sodium chloride concentration on DEAE-cellulose before (that is, omitting the urea-lithium chloride treatment) and after the removal of the oligo d(A-T), suggesting that the oligonucleotide was not interfering with this property of S factor. The Sephadex G-75 elution pattern of $d(A\text{-}T)_n \cdot d(A\text{-}T)_n$ - free S factor would be required to establish whether or not S factor is dimeric.

The S factor is distinguishable from the E. coli single-stranded DNA binding protein by its molecular weight in SDS-acrylamide gel electrophoresis. The S factor has a molecular weight of 11,500; the E. coli DNA binding protein, a molecular weight of 22,000 (44). In addition, preliminary experiments at 2.5 mM sodium phosphate, pH 7.2, have indicated that at a ratio of 7:1 (on a µg basis) of protein to DNA, S factor had no effect on the T_m of T4 DNA. At a similar protein to DNA

ratio and a slightly lower ionic strength, the E. coli single-stranded DNA binding protein lowers the T_m of T4 DNA (44).

Fraction 7 and Fraction 8 S factor can be used interchangeably for the synthesis of polymers. Fraction 7, however, does contain traces of nuclease activity which may stimulate synthesis. The failure to reduce the CLC DNA content of polymers below 5% with high levels of either Fraction 7 or 8 (Figure 16) may be a reflection of some inherent structural feature of the template.

CHAPTER V

EFFECTS OF S FACTOR ON THE SYNTHETIC REACTION

I. Introduction

There have been several reports of proteins other than nucleases which affect DNA synthesis catalyzed by DNA polymerases *in vitro*. The T4 gene 32 protein (32), *E. coli* single-stranded DNA binding protein (44), T7 single-stranded DNA binding protein (45), avian myeloblastosis virus (AMV) DNA polymerase stimulating protein (92), and copolymerase III* (102) all stimulate *in vitro* DNA synthesis catalyzed by their respective polymerases (Table IX).

TABLE IX

DNA POLYMERASE STIMULATING PROTEINS

Protein	Polymerase	Reference
T4 gene 32 protein	T4 DNA polymerase	(32)
E. coli single-stranded DNA binding protein	*E. coli* DNA polymerase II	(44)
T7 single-stranded DNA binding protein	T7 DNA polymerase	(45)
AMV stimulatory protein	AMV DNA polymerase	(92)
Copolymerase III*	*E. coli* DNA polymerase III* (an altered form of *E. coli* DNA polymerase III)	(102)

The exact mechanism of stimulation may involve the interaction of protein with its respective polymerase, since the protein is specific for its polymerase.

This chapter summarizes the effects of S factor on the DNA synthesis catalyzed by E. coli DNA polymerase I in vitro.

II. Results

A. Effect of S Factor on CLC DNA Production in $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$

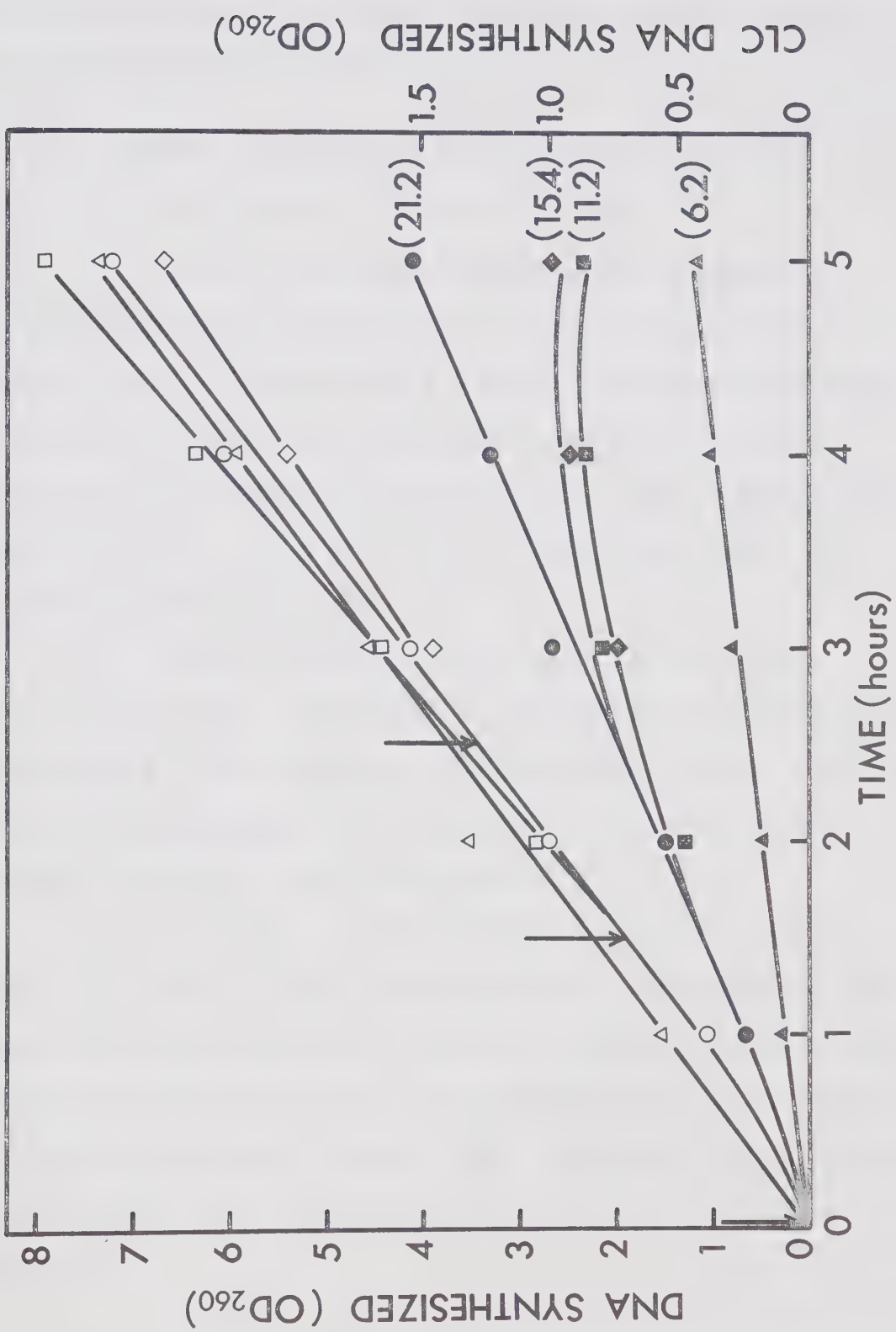
Separable-stranded $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ was used as a template for DNA polymerase I reactions in the absence or presence of 7.5 µg/ml Fraction 7 S factor. Fraction 7 was added at 0 time or after synthesis had proceeded for 1.5 or 2.5 hours. Portions of the incubation mixtures were removed at various times and assayed for CLC sequences or total bihelical DNA by the fluorescence method (Figure 21). When S factor was present from time 0, the CLC DNA was low. In the absence of S factor, there was a significant production of CLC DNA which was not removed by the addition of S factor at 1.5 or 2.5 hours. The actual amount of CLC remained constant after addition at these times, whereas, the percentage of the DNA which was CLC decreased.

B. Incubation of CLC $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ with S Factor

To determine whether S factor could remove CLC structures, CLC $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$, made in the absence of S factor, was treated with 50 µg/ml of either Fraction 7 or Fraction 8 S factor. In two cases, 12 mM $MgCl_2$ was also present. Portions were removed at various times and assayed by the fluorescence assay for total bihelical DNA or CLC

FIGURE 21. Production of CLC $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ in the Presence of S Factor.

Polymers were synthesized under standard conditions in the absence or presence of 7.5 µg per ml Fraction 7 S factor. As indicated by the arrows, Fraction 7 was added at 0, 1.5, or 2.5 hours. Portions were removed from the synthesizing mixtures and total DNA synthesized (open symbols) or CLC DNA synthesized (closed symbols) was determined by the fluorescence assay. The numbers in brackets indicate the percentage of the newly made DNA which was CLC. Circles, no Fraction 7; triangles, Fraction 7 at 0 time; squares, Fraction 7 at 1.5 hours; diamonds, Fraction 7 at 2.5 hours.


DNA SYNTHESIZED (OD260)
CLC DNA SYNTHESIZED (OD260)
TIME (hours)
(21.2)
(15.4)
(11.2)
(6.2)

DNA (Figure 22). The S factor did not remove CLC sequences. When nuclease was present (Fraction 7 plus $MgCl_2$), there was nucleolytic degradation but no preferential removal of CLC sequences.

C. Physical Properties of $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ Synthesized in the Absence or Presence of S Factor

Isolated polymers were synthesized from separable-stranded $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ under standard conditions in the absence or presence of Fraction 7 or Fraction 8 S factor. Their molecular weights were determined by the Studier method (79), and their CLC content determined by the fluorescence method (Table X). There were two main effects of S factor. These were on the CLC DNA content and on the single-stranded molecular weight.

Polymers made with S factor had a low CLC content compared to those made in its absence. Alkaline CsCl equilibrium density banding confirmed this observation. Optical density patterns similar to Figure 6a for polymer made in the absence of S factor, and to Figure 6b for polymer made with S factor were obtained.

The alkaline molecular weights for polymers made with S factor were lower than those for polymers made in its absence. For polymers made with S factor, the ratio of the alkaline molecular weight to the neutral molecular weight was about one-half the ratio observed in the polymers made without S factor. This ratio for CLC DNA is consistent with a strand-switching mechanism for CLC production, as discussed in Chapter VI.

FIGURE 22. Incubation of CLC $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ with Fraction 7 or Fraction 8 S Factor.

The incubation mixture contained 30 mM potassium phosphate, pH 7.4, 2 mM dithiothreitol, 1 OD_{260} CLC $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$, 0.5 OD_{260} tRNA, 50 µg/ml either Fraction 7 or Fraction 8, and where indicated 12 mM $MgCl_2$. Incubations were at 37°. Portions were removed at various times and total DNA (open symbols) or CLC DNA (closed symbols) was determined by the fluorescence assay. The numbers in brackets indicate the per cent of the total DNA which was CLC. Circles, Fraction 7; triangles, Fraction 7 plus $MgCl_2$; squares, Fraction 8 plus $MgCl_2$.

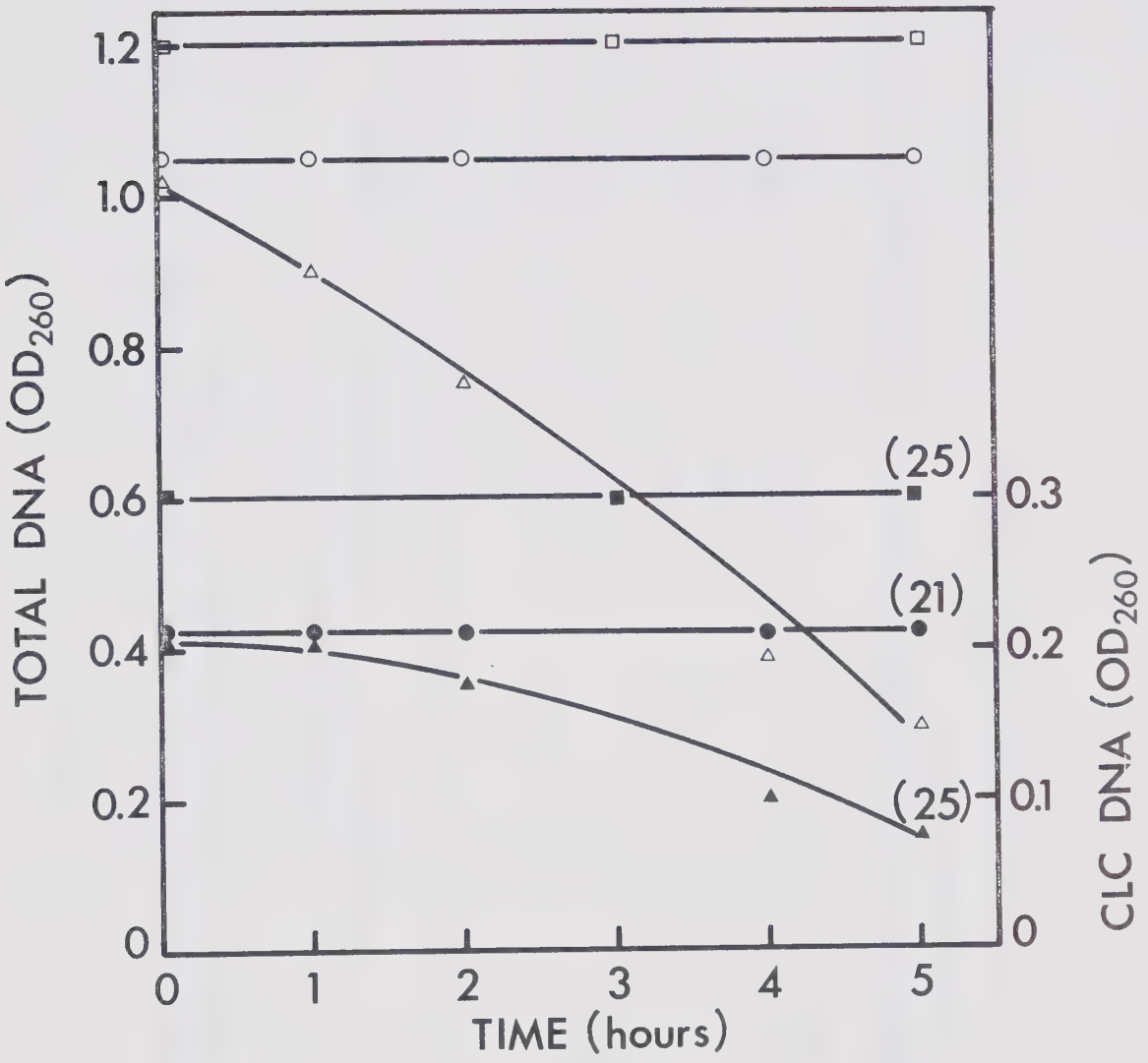

1.2
1.0
0.8
0.6
0.4
0.2
0
TOTAL DNA (OD260)
0.3
0.2
0.1
0
CLC DNA (OD260)
(25)
(21)
(25)
0
1
2
3
4
5
TIME (hours)

TABLE X

MOLECULAR WEIGHTS AND CLC CONTENT OF $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ POLYMERS SYNTHESIZED IN THE ABSENCE OR PRESENCE OF S FACTOR

Conditions of Synthesis	Molecular Weight x 10^{-3} [a] Single-stranded (SS)	Double-stranded (DS)	Ratio (SS)/(DS)	% CLC [b]
- S Factor	259	378	0.69	20.9
- S Factor	249	384	0.65	22.0
AVERAGE	254	382	0.67	21.5
+ 5 µg/ml Fraction 8 S Factor	135	370	0.37	6.7
+ 7.5 µg/ml Fraction 7 S Factor	133	356	0.38	6.9
+ 5 µg/ml Fraction 8 S Factor	121	423	0.29	7.1
AVERAGE	130	383	0.34	6.9

[a] Molecular weights were determined under native (double-stranded) or alkali denatured (single-stranded conditions by the Studier method (79).

[b] The fluorescence method was used to determine the CLC DNA content.

D. Effect of S Factor on *E. coli* DNA Synthesis *In vitro*

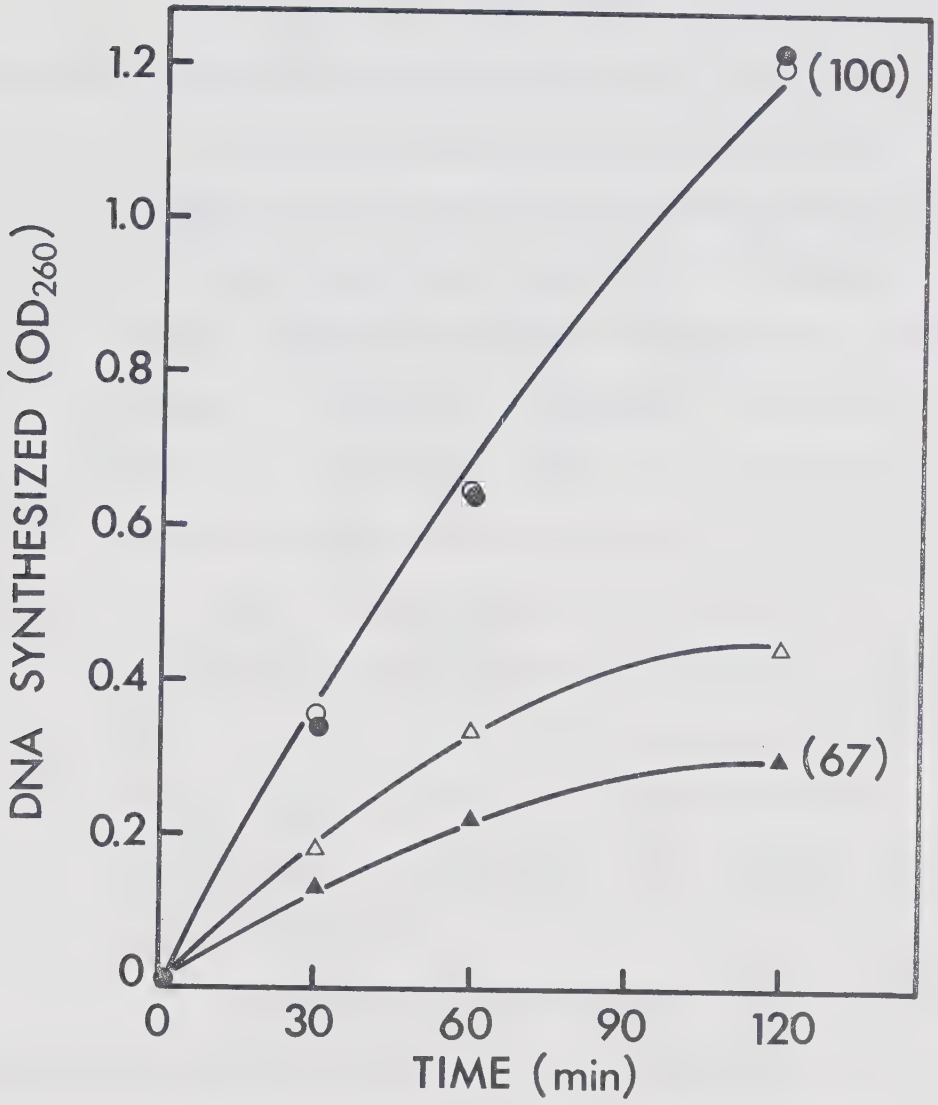
E. coli DNA, 0% CLC, at 0.5 OD_{260} was used as a template for a DNA polymerase I reaction in the absence or presence of 5 µg/ml Fraction 8 S factor. Portions were removed at various times and analyzed by the fluorescence method using the KE buffer system to determine the amount of DNA synthesized and its CLC content (Figure 23). In the presence of S factor, the synthesis was inhibited, and the per cent CLC of the product was reduced to 67. Similar results were obtained with 20 µg/ml Fraction 8 S factor.

III. Discussion

The major affect of S factor on the defined polymer $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ was the prevention of CLC DNA formation during synthesis. For this effect however, S factor had to be present at 0 time. Once a CLC linkage had been formed it was resistant to the action of S factor. This S factor activity is not restricted to the $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ polymer. Recent work has indicated that S factor also prevents the formation of CLC DNA during the synthesis of the defined polymer $d(T\text{-}T\text{-}G)_n \cdot d(C\text{-}A\text{-}A)_n$ (Coulter, M., Flintoff, W., Paetkau, V., Pulleyblank, D., and Morgan, A.R.: manuscript submitted).

The effect of S factor during the synthesis using a natural template was two fold. In addition to partially reducing the CLC DNA content, net-fold synthesis did not occur. Both of these findings would be consistent with a block in the major synthesis mechanism, strand displacement (Chapter VI). The complexity of the DNA and perhaps the requirement of the system for additional components may

FIGURE 23. Effect of S Factor on *E. coli* DNA Synthesis *In Vitro*. *E. coli* DNA was synthesized under standard conditions in the absence or presence of 5 μg/ml Fraction 8 S factor. The initial template, 0% CLC, was 0.5 OD_{260}. Portions were removed at various times and analyzed in the KE buffer system for total bihelical DNA synthesized (open symbols) or CLC DNA synthesized (closed symbols). The results are expressed in terms of the newly made DNA. The numbers in brackets indicate the percentage of the newly made DNA which was CLC. circles, no S factor; triangles, 5 μg/ml Fraction 8 S factor.


DNA SYNTHESIZED (OD_{260})
1.2
1.0
0.8
0.6
0.4
0.2
0
0
30
60
90
120
TIME (min)
(100)
(67)

explain the limited effect that S factor had on the prevention of CLC DNA formation using the natural template.

Several observations using synthetic polymers suggest some specificity for the action of S factor. These are:

(a) The S factor was unable to remove CLC DNA sequences once they were formed (Figure 21,22).

(b) The S factor was effective throughout the synthesis despite an increased concentration of DNA (Figure 21); therefore, S factor may be specific for the sites of synthesis, which would not increase in direct proportion to the total DNA.

(c) In the presence of S factor, DNA polymerase appeared to selectively copy non-CLC DNA when both CLC and non-CLC DNA were present (Figure 21 - The absolute level of CLC DNA present remained constant, but the percentage of the total DNA which was CLC decreased.).

In addition to the requirement for S factor at 0 time, the production of separable-stranded DNA <u>in vitro</u> requires that the template have certain physical properties. Heating and cooling a $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ polymer produced a highly branched structure (Paetkau, V.: unpublished electron micrographs). Such a polymer, when used as a template in the presence of S factor, led to the synthesis of DNA containing CLC sequences (Coulter, M., Flintoff, W., Paetkau, V., Pulleyblank, D., and Morgan, A.R.: manuscript submitted). Thus, S factor will prevent the production of DNA with CLC sequences if the template for the reaction is of a simple, linear nature, with separable strands.

CHAPTER VI

CONCLUSION

A model for *in vitro* DNA synthesis has been postulated by Schildkraut, Richardson, and Kornberg (61) to explain the synthesis catalyzed by *E. coli* DNA polymerase I. This mechanism explains the production of CLC DNA (Figure 24).

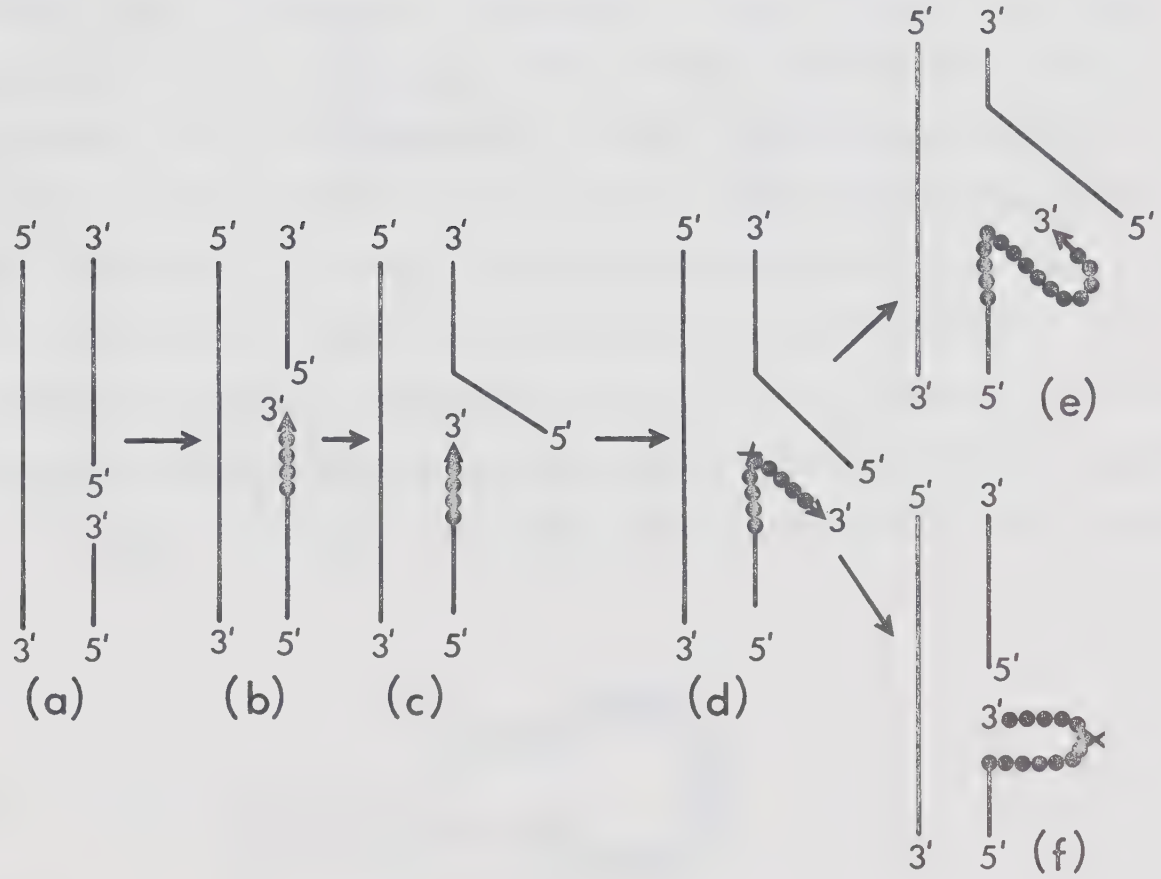


FIGURE 24. Strand Displacement Mechanism for DNA Synthesis.
——— , parental; ●●●●● , newly made.

Synthesis begins at a nick (Figure 24a) by the addition of deoxynucleotide residues onto the 3'-hydroxyl terminal of the parental primer. The initial phases of the synthesis may involve a repair-type reaction in which the nick is translated toward the end of the molecule (Figure 24b). At some point during the synthesis, the 5' strand is displaced (Figure 24c). After further copying, the polymerase molecule may switch strands and begin to copy this displaced strand (Figure 24d). The result is CLC DNA. That such a mechanism does occur with E. coli DNA polymerase I acting on a circular, natural DNA template in vitro is demonstrated by the work of Masamune and Richardson (93). Subsequent to strand displacement, the newly made DNA may loop back on itself to form a "hairpin" structure (104), or the polymerase molecule may turn around. In either case, the polymerase molecule uses the newly made DNA as template for additional synthesis (Figure 24e). The result is CLC DNA and a branched structure (93,103). Branched structures however, could also arise by strand switching followed by branch migration (105) (Figure 24f). Calf thymus DNA polymerase synthesizes CLC structures

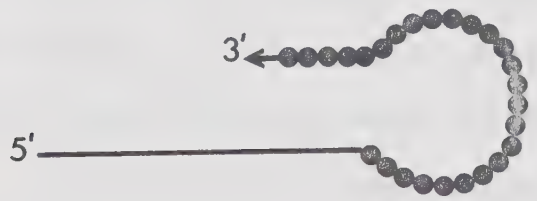


FIGURE 25. Formation of CLC Sequences by Calf Thymus DNA Polymerase. ——— , parental; ●●●●● , newly made.

probably by a turning-around mechanism. This enzyme can add deoxynucleotide residues onto the 3'-end of a single-stranded DNA, forming hairpin structures entirely of newly made DNA (59) (Figure 25).

A slippage mechanism for DNA synthesis does not give rise to CLC DNA (Figure 26).

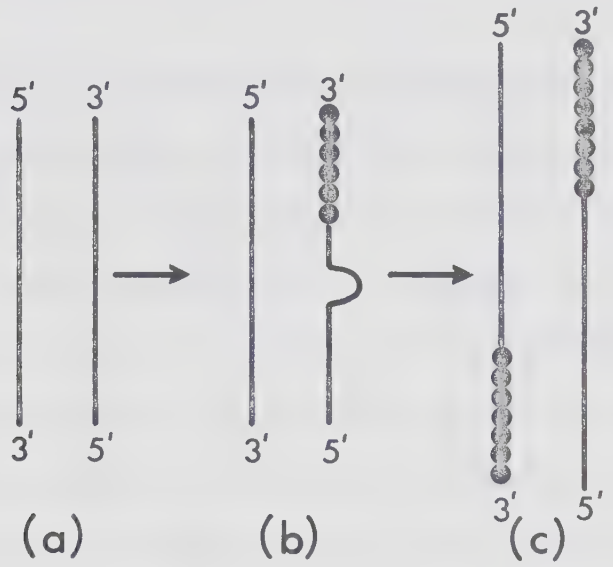


FIGURE 26. Slippage Mechanism for DNA Synthesis.
——— , parental; ●●●●●● , newly made.

The molecule "breathes" in such a way as to allow repair-type synthesis to occur on one strand (Figure 26b). The molecule then relaxes and repair synthesis occurs on the other strand (Figure 26c).

A strand-switching mechanism may be occurring in the synthesis of certain defined DNAs in our work since CLC DNA was produced and the single-stranded molecular weight of such DNA was greater than one-half the double-stranded molecular weight (Table X). Since all the newly synthesized DNA was not CLC, slippage may also be occurring. This is supported by the finding that the repeating tri-

nucleotide polymer $d(T\text{-}T\text{-}G)_n \cdot d(C\text{-}A\text{-}A)_n$, which is less likely to slip than the repeating dinucleotide polymer $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$, has a 10-20% higher CLC DNA content than $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ (Coulter, M., Flintoff, W., Paetkau, V., Pulleyblank, D., and Morgan, A.R.: manuscript submitted). Slippage may be the mechanism whereby polypyrimidine·polypurine DNAs are synthesized.

Since strand-switching or turn-around is probably the mechanism for synthesis from a natural DNA template in vitro, it would be interesting to determine whether there are unique sites on the chromosome where such "turn-arounds" occur. A template for this would be relaxed colicin E1 DNA which contains a single, specific nick (106). The DNA would be copied with E. coli DNA polymerase I in vitro. After partial degradation with endonuclease I, the product would be denatured and chromatographed on hydroxylapatite. Since the CLC region would be double-stranded, it would be separated from the single-stranded DNA. The CLC region could then be sequenced by a series of nuclease digestions.

If CLC DNA is a result of strand displacement and strand-switching, how is S factor preventing the accumulation of such structures during synthesis? There are two possibilities:

(1) The S factor may be acting as a nuclease specific for the "looped-out" region at the CLC sequence linker (107). Although there was no demonstrable nuclease activity in purified S factor, the nuclease assays may have been limited by the templates used. To demonstrate such a specific nuclease activity would require that the template be similar to the structure of DNA generated during synthesis. A candidate for

such a template might be a "hairpin" DNA structure. However, molecules of the CLC type were not converted to separable strands by S factor, making this an unlikely mechanism.

(2) The S factor may be preventing strand-switching by the polymerase by binding to the displaced strand. The finding that S factor was associated with low molecular weight oligo d(A-T) suggests that it readily binds to nucleic acids. In the presence of S factor, the inhibition of E. coli DNA synthesis and the reduction in CLC would be consistent with a block in the strand-switching mechanism of synthesis.

Further work will be required to differentiate between these proposed mechanisms and to establish whether the activity of S factor involves an association with nucleic acid, or polymerase, or both.

An additional template for S factor would be a circular, nicked DNA. When this DNA (for example, nicked PM2 DNA, and, perhaps, colicin E1 DNA) is copied in vitro by E. coli DNA polymerase I, it generates rolling circles, with one strand continuously being displaced, and forms CLC DNA (93). The effect of S factor on CLC DNA formation during synthesis of these DNAs, and the physical structures of the resulting products could be easily tested. The DNA synthesis using natural DNAs as templates in the presence of S factor may provide a system to test the involvement of additional components. Since net-fold synthesis was inhibited various components which stimulate synthesis could be tested. Such a system would be analogous to the ϕX174 DNA in vitro synthesizing

system described by Hurwitz, Wickner, and Wright (108). In this, a factor was demonstrated to restore dependence on E. coli RNA polymerase to the copying of ϕX174 DNA by E. coli DNA polymerase III.

The in vitro production of CLC DNA has not been well established, and whether or not S factor has an in vivo role is, of course, also questionable. Several approaches may be used to determine whether these components are of in vivo significance:

(1) E. coli strains thermosensitive for DNA synthesis in the loci dna A, B, C, D, and G are not yet biochemically characterized. The sudden production of CLC DNA after switching to the nonpermissive temperature in these strains would be evidence for an S factor activity at these loci in vivo. The best candidates are B and C (D) since A is involved with initiation of synthesis (109) and G has a molecular weight of 60,000 (38). Biochemical (37) and genetic (110) evidence suggest that dna C and dna D gene products may be related. One of the activities of C (D) in an in vitro complementation assay has a molecular weight of 25,000 (37) which is similar to the molecular weight of S factor.

(2) E. coli treated with toluene and Triton X-100 are permeable to antibodies (51). An antibody to purified S factor might then be used to inactivate the protein in the cell, and CLC DNA

measured. Alternately, permeable cells may already produce significant levels of CLC DNA due to a partial loss of S factor; in that case, addition of exogenous S factor might reduce the level of CLC DNA. The Cellophane disc system (53) could function similarly.

Although there is no genetic evidence for the involvement of S factor in replication, interest is maintained by the fact that when it is present in a DNA polymerizing reaction several identical copies of a defined, separable-stranded DNA of high molecular weight are made. This is a simple model for the DNA synthesis occurring during replication.

Further investigations of the effect of S factor on the alkaline molecular weights of various polymers may give a clue to the mechanism of synthesis. Models similar to that proposed by Morgan (111) would predict that non-CLC DNA would have an alkaline molecular weight one-half that of CLC DNA.

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